The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

A study of the relationship among *all-trans* retinoic acid, arginase I and apoptosis: implication of arginase I in controlling cell death

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A thesis submitted

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

August 2009

Certificate of Originality

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Chow Ho Yin

August 2009

Abstract of the thesis entitled 'A study of the relationship among *all-trans* retinoic acid, arginase I and apoptosis: implication of arginase I in controlling cell death',

Submitted by Chow Ho Yin

For the degree of Doctor of Philosophy

At the Hong Kong Polytechnic University in August 2009.

Name of supervisor: Dr. Leung Yun Chung, Thomas

All-*trans*-retinoic acid (RA) is able to induce cell growth inhibition or apoptosis in many kinds of malignant cells such as hepatocarcinoma, melanoma and leukemia. Previously, our collaborators found that when RA was injected into pregnant mice, the embryos showed caudal regression due to apoptosis in the early stage of embryogenesis. Just right before apoptosis took place, we found by differential display that the arginase I gene was up-regulated 5 h after RA was injected. Therefore, we hypothesized that RA is able to induce arginase I up-regulation directly which consequently stimulates apoptosis in some cells. In this thesis, we aimed to find out whether arginase is involved in RA-induced cell death or cell growth inhibition in mouse embryonic carcinoma cells P19 and melanoma B16-F0 (B16) cells, and the possible occurrence of the retinoic acid response element (RARE) in the promoter region of the mouse arginase I gene. In addition, the role of arginase, either intra-cellularly induced or extra-cellularly added, would be discussed. Both P19 and B16 cells were exposed to different concentrations of RA for 24-72 h. Arginase mRNA expression was

detected by real-time PCR and its enzyme activity by activity assay. Cell growth inhibition was measured by MTT viability assay. Apoptosis of cells was measured by Annexin V-FITC apoptosis staining kit using flow cytometry. After exposure to RA, both P19 and B16 cells showed significant up-regulation of arginase I mRNA expression, but not arginase II, in a time- and dose-dependent manner, and the elevated arginase activity induced by RA paralleled with cell growth inhibition of both cell lines. In P19 cells, arginase I mRNA was induced in 12 h but apoptosis was stimulated in 18 h. Surprisingly, RA stimulated time-dependent apoptosis in P19 cells but only induced time-dependent growth inhibition without apoptosis in B16 cells. To test the arginase dependency of RA-induced apoptosis in P19 cells and cell growth inhibition in B16 cells, arginase inhibitor was added together with RA and apoptosis and cell viability assay was performed. Apoptosis was reduced when N^{ω}-hydroxy-nor-arginine (norNOHA) was added together with RA in P19 cells. Cell growth was restored when arginase inhibitor norNOHA or N^{ω}-hydroxy-*L*-arginine (NOHA) was added in addition to RA in B16 cells. *L*-ornithine and polyamines, the products of arginase-ornithine decarboxylase (ODC) pathway, could also induce either apoptosis in P19 cells or cell growth inhibition in B16 cells. RA-induced apoptosis in P19 cells was abolished by inhibition of ODC using specific ODC inhibitor α-Difluoromethylornithine (DFMO) but was restored by polyamines, indicating the effectors in the relationship between RA, arginase and apoptosis or cell growth inhibition in the two cell lines.

The data of the reporter assay using luciferase system showed that there was a consensus RARE sequence located in the promoter region of mouse arginase I gene. In addition, arginase I mRNA was still up-regulated by RA in the presence of protein synthesis inhibitor cycloheximide (CHX), indicating that RA induces arginase I transcription directly and requires no *de novo* protein synthesis.

Since arginase was up-regulated by RA and stimulated apoptosis in P19 cells, we tested whether arginase induced by other molecules would result in apoptosis as well. Cyclic AMP (cAMP), a well-known and patented arginase-inducing agent, was added to P19 cells for qRT-PCR assay, arginase activity assay and apoptosis assay. Consistent with other published data, arginase activity was up-regulated in P19 cells except that both arginase I and arginase II mRNA levels were found to account for the up-regulation of activity. To check if cAMP-induced apoptosis in P19 cells was arginase-dependent, cAMP was added to the cells in the presence of norNOHA. Apoptosis was abolished by the inhibition of arginase, similar to RA-induced apoptosis, indicating that once arginase was up-regulated in P19 cells, cells would undergo apoptosis.

Besides intra-cellular arginase gene induction, arginase protein was added to the growth medium of P19 and B16 cells to check if there would be apoptosis and cell growth inhibition. After bovine arginase was added to the of P19 cells for 24 h, arginine in the culture medium was greatly reduced with the elevation of ornithine. Moreover, we observed cell cycle arrest in the S phase and the G_0/G_1 phase of P19 and B16 cells, respectively. Both cell lines showed

apoptosis. Cell death was induced by depleting arginine in the growth medium because the cells were found to depend on the availability of arginine in the growth medium.

For the first time, these data suggest that both intra-cellular and extra-cellular arginase stimulate apoptosis in P19 cells or growth inhibition in B16 cells. The expression modulation of arginase may provide insight into treatment of arginase-related diseases.

Acknowledgements

I am grateful to my supervisor Dr. Thomas Y. C. Leung for his excellent guidance, valuable advice and expertise. He taught me the passion, emotion and enthusiasm of the research science. I would also like to express my thanks to Dr. Alisa S. W. Shum. The advice that I received from her was instrumental in helping me to finish my thesis. I am also thankful to Dr. Daniel K. L. Lee who provided the luciferase reporter plasmids, which were important materials for the whole project.

I would also like to particularly thank to Dr. Alan T. L. Lam and Dr. Gabriel K. Y. Wong for their knowledge and advice. I am grateful to my research group members, Dr. Christine S. Y. Kwok, Dr. H. K. Yap, Dr. Emily M. W. Tsing, Dr. Christina K. W. To, Dr. P. K. So, Miss Sammi S. M. Tsui, Miss Kareena M. S. Tsang, Mr. Angus C. L. Chan, Miss Sandra Y. S. Siu, Mr. Steve H. C. Chong and Miss W. T. Wong. Interacting with them always brightened my day.

I would also like to express my thanks to the technicians of the Department of Applied Biology and Chemical Technology, especially Dr. Mabel Y. C. Yau and Miss Sarah K. L. Yeung, for their technical assistance.

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3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	MTT
Adenosine 3′,5′-cyclic monophosphate	cAMP
All-*trans*-retinoic acid	RA
Arginase I	ArgI
Arginase II	ArgII
Arginine decarboxylase	ADC
Bacterial artificial chromosome	BAC
Bovine serum albumin	BSA
Cellular retinoic acid binding protein	CRABP
Cycloheximide	CHX
Cyclic AMP response element	CRE
Dexamethasone	DEX
α-Difluoromethylornithine	DFMO
Dimethyl sulfoxide	DMSO
Direct repeat	DR
Fatty-acid binding protein	FABP
Fetal bovine serum	FBS
Hepatocellular carcinoma	HCC
Inducible nitric oxide synthase	iNOS
Interferon-γ	IFN-γ
Lipopolysaccharide	LPS

Myelin-associated glycoprotein	MAG
N^{ω}-hydroxy-*L*-arginine	NOHA
N^{ω}-hydroxy-nor-arginine	norNOHA
N^6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate	dbcAMP
Nitric Oxide	NO
Ornithine aminotransferase	OAT
Ornithine decarboxylase	ODC
Ornithine transcarbamoylase	OTC
Pegylated recombinant human arginase	rhArg-PEG
Phosphate-buffered saline	PBS
Putrescine	PUT
Propidium iodine	PI
Quantitative reverse transcription – polymerase chain reaction	*q*RT-PCR
Rat aortic smooth muscle cells	RASMC
Retinoic acid receptor	RAR
Retinoic acid response element	RARE
Retinoid X receptor	RXR
Semi-quantitative reverse transcription – polymerase chain reaction	RT-PCR
Spermidine	SPD
Spermine	SP
Tris-borate-EDTA	TBE

1 Introduction

Arginase (*L*-arginine ureahydrolase, or amidinohydrolase, EC 3.5.3.1) is a hydrolytic enzyme that is responsible for the conversion of arginine, a non-essential amino acid, into ornithine and urea. It is very important for cells to function properly. The product, ornithine, is of great importance in polyamine synthesis, which eventually leads to cell proliferation by the binding of polyamines to nucleic acids, influencing their conformation, rate of synthesis and fidelity of information transfer (Pegg and McCann 1982; Jenkinson et al. 1996). Arginine is also utilized by other biochemical pathways such as nitric oxide synthase (NOS) to form nitric oxide (NO), which plays an important role in apoptosis (Iwashina et al. 1998). Besides, arginine is directly used in protein synthesis.

Arginase's relationship to cell proliferation, growth inhibition and cell death has long been investigated. Arginase has at least two isomer forms: arginase I (hepatic arginase) and arginase II (extrahepatic arginase). Arginase I can mostly be found in the cytoplasm of liver. It is one of the five urea cycle enzymes and is responsible for the detoxification of ammonia and production of urea. It takes part in the conversion of arginine, which is produced from argininosuccinate by argininosuccinate lysate (ASL), into ornithine and urea. Urea is excreted, whereas ornithine is directed towards polyamine synthesis by ornithine decarboxylase (ODC) (Jenkinson et al. 1996). Arginase II is located in the mitochondrial matrix of various tissues. It was proposed to take part in

many biosynthetic pathways, such as polyamine synthesis (Russell and McVicker 1972), proline and glutamate synthesis (Yip and Knox 1972), γ-aminobutyric acid formation (Johnson and Roberts 1984), immune system function (Currie 1978) and nitric acid synthesis (Cook et al. 1994; Daghigh et al. 1994; Corraliza et al. 1995).

1.1 Regulation of cell growth and cell death by arginase in cells

1.1.1 Effect of arginase on cell proliferation

Arginase (arginase I and arginase II) catalyzes the conversion of arginine into ornithine and urea. Ornithine is an important molecule in many biological functions and acts as a common substrate for ornithine decarboxylase (ODC), ornithine acetyltransferase (OAT) and ornithine transcarbamylase (OTC). In some cases, ornithine is further catalyzed to putrescine (the precursor of other polyamines) by ODC (Gillette and Mitchell 1991). Putrescine and decarboxylated S-adenosylmethionine act as the substrates for the synthesis of spermidine and further to spermine. Polyamines (putrescine, spermidine and spermine) are of great importance in cell cycle progression, and in turn, for cell proliferation by binding to nucleic acids (Pegg and McCann 1982; Jenkinson et al. 1996). Therefore, once arginase is up-regulated, the elevated levels of ornithine will consequently lead to the accumulation of polyamines, thus enhancing the proliferation of the arginase-up-regulated cells. Wei et al. (2001) showed that when the rat arginase I gene was transfected into rat aortic smooth muscle cells (RASMC), the selected population, which expressed a 10-fold elevation of arginase enzyme activity, showed an over 60 % increase in the rate of cell proliferation as determined by thymidine incorporation into DNA. The reason for the increase in cell proliferation was that the over-expression of arginase I resulted in an elevation

of ornithine and consequently the accumulation of polyamines, and the increased level of polyamines promoted the proliferation of cells because once arginase activity was inhibited by arginase inhibitors, the cell proliferation of the arginase I-transfected RASMC decreased with a decrease in polyamine production.

1.1.2 Effect of arginase on reducing apoptosis

Arginine is a non-essential amino acid that can be self-reproduced through the urea cycle in the hepatocytes. Being the most common amino acid in the body, one of its main functions is the protein synthesis. Many apoptotic events often require new protein synthesis (Martin et al. 1988; Pittman et al. 1993). Therefore, Esch et al. (1998) hypothesized that cells could be protected from apoptosis by depleting arginine available in the growth medium. In neuronal cells, when apoptosis was induced by glutathione depletion or Sindbis virus infection, arginase could protect the cells from apoptosis. It was shown that the neuronal apoptosis-inhibiting effect of arginase was nitric oxide- (NO) independent. In addition, protein synthesis was greatly reduced and paralleled with the concentration-dependent addition of arginase into N18 neuroblastoma cells. Therefore, it was concluded that neuronal apoptosis was inhibited by arginase in a mechanism wherein arginase depleted arginine in the culture medium, and the decreased level of arginine consequently resulted in a decrease in protein synthesis required for apoptosis. In addition, the decreased level of arginine could also lead to a down-regulation of NO production, which had been shown to induce apoptosis in some neuronal cells.

Besides neuronal cells, a similar phenomenon was observed in murine macrophage in 1999. Gotoh and Mori (1999) found that in murine macrophage-derived RAW 264.7 cell, inducible nitric oxide synthase (iNOS) stimulated by lipopolysaccharide (LPS) and interferon-γ (IFN-γ) resulted in an accumulation

of NO which was responsible for the apoptosis of the cells. It was also proved that when arginase II was induced by dexamethasone (DEX) and cyclic AMP (cAMP) in activated RAW 264.7 cells, there was no iNOS suppression, but apoptosis was abolished with the limited production of NO. Moreover, when either arginase I or arginase II was transiently transfected, RAW 264.7 cells were rescued from LPS/ IFN-γ-induced apoptosis. In addition, a NO donor or a high concentration of arginine would totally cancel out the anti-apoptotic effect of up-regulated arginase by induction or transfection. These data had shown that arginase functioned by depleting arginine available for the production of NO by iNOS, therefore limiting the level of NO and consequently decreasing the apoptosis stimulated by NO (Gotoh and Mori 1999).

1.1.3 Effect of arginase on overcoming growth inhibition

Wei et al. (2001) described that the transfection of arginase into RASMC induced an increase in cell proliferation by accumulating polyamines through the conversion of ornithine, produced from arginase, into putrescine by ODC, and then into spermidine and spermine. A similar observation was reported by Cai et al. (2002). This research group found that adding cAMP would result in the overcoming of inhibition by myelin-associated glycoprotein (MAG) and myelin in the cerebellar and DRG neurons. The underlying mechanisms were that cAMP induced the up-regulation of arginase I mRNA expression, which eventually led to the accumulation of putrescine, similar to the observation by Wei et al. (2001). Cai et al. (2002) also showed that either the transfection of arginase I or the elevating of putrescine was sufficient to overcome the inhibition of regeneration by MAG and myelin, indicating the role of arginase in converting arginine into ornithine and eventually into polyamines by ODC, and the proliferating effect of polyamines.

1.1.4 Effect of knocking out the arginase gene on cell proliferation

All of the previous reports described above concluded that arginase was beneficial to cells ranging from RASMC, murine macrophage RAW 264.7 cells, neuronal cells, neuroblastoma cells, cerebellar to DRG neurons. It stimulated cell growth by producing more ornithine from arginine, thus providing more polyamines for cells. The polyamines then promoted cell proliferation (Wei et al. 2001), protected cells from apoptosis (Esch et al. 1998; Gotoh and Mori 1999) and from growth inhibition (Cai et al. 2002). However, Becker-Catania and Gregory (2006) found that the expression of arginase I gene was not beneficial to neural stem cells isolated from the germinal zones of embryonic or newborn animals. They showed that the proliferation rate of arginase I knocked-out neural stem cells was higher than that of the wild-type and heterozygous control cells in both short term and long term incubation. They suggested that the increased proliferation was related to the polyamines formation, not by the arginase-ODC pathway because ODC was found down-regulated in the arginase I knocked-out neural stem cells, but through the up-regulated arginine decarboxylase (ADC) and agmatinase, which converted arginine into agmatine and then into putrescine. In addition, the expression level of spermine synthase was found to have increased, indicating an up-regulation of the production of spermidine from putrescine. This was further confirmed by the reduction in spermine-spermidine acetyltransferase levels which reflected that spermine-to-spermidine and spermidine-to-putrescine

conversion were down-regulated (Becker-Catania et al. 2006).

1.1.5 Effect of arginase on cell death

Arginase was reported to be important in regulating cell growth or protecting cells from apoptosis, and all the above had mentioned that these effects were related to the cell proliferating property of polyamines. It was recently reported that arginase was involved in cell growth inhibition or cell death in some cells. For example, up-regulation of arginase II by *Helicobacter pylori* (*H. pylori*) infection caused apoptosis in murine macrophage RAW 264.7 cells. Gobert et al. (2002) reported that *H. pylori* stimulated arginase II mRNA expression and the corresponding arginase enzyme activity, and the macrophage apoptosis was parallel with the arginase activity. The *H. pylori*-stimulated macrophage apoptosis was reverted in the presence of arginase inhibitor and ODC inhibitor, showing that the *H. pylori*-stimulated macrophage apoptosis was arginase- and ODC-dependent. Moreover, they proved that spermidine and spermine restored the *H. pylori*-stimulated macrophage apoptosis in the presence of ODC inhibitor, and spermidine and spermine induced apoptosis in macrophage cells, indicating the role of polyamines in macrophage apoptosis. Although iNOS was also up-regulated, adding NOS inhibitor did not rescue the macrophage from apoptosis (Gobert et al. 2002). Arginase II is a mitochondria enzyme that would preferentially promote proline and glutamate synthesis from ornithine because OAT is also located in the mitochondria (Wu and Morris 1998). However, another report showed that proline could be converted into ornithine (Wu and Morris 1998) and ornithine

can be transported from the mitochondria to the cytoplasm where ODC is present to convert ornithine into polyamines (Indiveri et al. 1997).

While arginase was induced intra-cellularly by *H. pylori* and caused apoptosis in macrophage cells, we previously found that adding arginase extra-cellularly in the culture medium of hepatocellular carcinoma (HCC) caused cell growth inhibition by inducing cell cycle arrest (Cheng et al. 2007; Lam et al. 2009; Tsui et al. 2009). Cheng et al. (2007) found that pegylated recombinant human arginase (rhArg-PEG) was able to stimulate growth inhibition in different HCC cell lines, such as Hep3B and HepG2, by depleting arginine in the growth medium. The underlying mechanisms were that the cells were deficient in expressing ornithine transcarbamylase (OTC), so the cells could no longer recycle arginine because ornithine could not be converted into citrulline by OTC in the urea cycle. Since in particular tumor cells, the cell cycle was defective in the 'R' checkpoint or other cycle aberrations, cells would continue the cycle instead of entering into quiescence (G_0) and this would result in an imbalance in nutrient and cell death in the condition of depleted arginine (Scott et al. 2000). Recently, Lam et al. (2009) further supported this observation by showing that citrulline could rescue the rhArg-PEG-induced growth inhibition in HCC Hep3B cells. The Hep3B cells were shown to express the urea cycle enzymes argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase, but not OTC. Therefore, it was proposed that in the patient bloodstream, rhArg-PEG depleted arginine and produced ornithine. When ornithine entered the HCC cells, it could not be converted into citrulline due to

the deficiency in the expression of OTC, therefore the HCC cells would be growth-inhibited (Lam et al. 2009).

Before the discovery of arginase to induce cell growth inhibition in HCC, Currie (1978) had already demonstrated that activated macrophages were able to kill tumor cells by releasing arginase into the surrounding environment. When peritoneal exudates macrophages were exposed to stimuli such as zymosan and LPS, arginase activity was detected either in the cells or in the culturing medium as early as 24 h. Arginase was produced and released, and exerted certain cytotoxicity to several kinds of tumor cells like V79 Chinese hamster lung cells, SL_2 and L5178Y lymphoma cells and HSN hooded rat sarcoma cells. The cytotoxic activity of the released arginase in the tumor cells could be prevented by the addition of arginine and citrulline but not ornithine or putrescine. Therefore, the cytotoxicity of the released arginase from activated macrophages on the target tumor cells was a consequence of arginine deprivation (Currie 1978).

The literatures described above suggested that the role of arginase is different in different kind of cells, and the effects may be different depending on whether the arginase is inside or outside the cells. Therefore, we hypothesize that the effect of arginase on cell proliferation, growth inhibition and cell death might greatly depend on the cell type and the surrounding environment.

1.2 Regulation of cell growth and cell death by *all-trans* retinoic acid (RA)

1.2.1 Introduction to RA

It has long been established that vitamin A and its derivatives, referred toas retinoids, are of great importance in the development and homoeostatsis in nearly all tissues by regulating the gene expression on cell differentiation, proliferation and cell death or apoptosis (Ross et al. 2000). There are already many reports showing that retinoids, especially RA, are able to induce cell growth inhibition or even cell death in many kinds of malignant cells such as mammary adenocarcinoma (Donato and Noy 2005; Donato et al. 2007), acute promyelocytic leukemia (Carpentier et al. 1998; Charrad et al. 2002; Otake et al. 2005), malignant melanoma (Desai et al. 2000; Emionite et al. 2003; Zhang et al. 2003), and hepatocellular carcinoma (Hsu et al. 1996; Kim et al. 1996; Arce et al. 2005). Recently, RA has been used as a chemotherapeutic agent for many kinds of malignant cancers.

1.2.2 Outline of RA metabolism

Retinoids can always be found in the human body and retinol (vitamin A) represents the most abundant retinoid in the blood while retinyl esters (mostly palmitate) represent the most abundant storage molecules in liver and other non-hepatic tissues (Napoli 1999). Normally retinoids can be absorbed from diet in the form of retinyl ester (retinyl palmitate) or β-carotene. Retinyl esters can be converted into retinol in a reversible reaction, and β-carotene is converted into retinal in an irreversible reaction. Retinol in the body can be reacted to retinal by retinol dehydrogenase, releasing the product *all-trans* retinaldehyde, which is further converted into *all-trans* retinoic acid (RA) by retinal dehydrogenase (Ross et al. 2000). RA then undergoes metabolism into 4-hydroxy-RA, 4-oxo-RA and 18-hydroxl-RA. These conversions act to eliminate RA by degradation, and are thought to control the retinoid signaling pathway. In addition, RA also undergoes isomerization in vivo into 9-*cis*-RA and 13-*cis*-RA, and the two isomers are able to bind with different kinds of receptors in the steroid hormone superfamily (Napoli 1999).

1.2.3 Mechanism of RA action

RA does not act directly to the DNA sequence to initiate gene regulation, but it is involved in a complicated but highly regulated and sequenced reaction. When entering into cells, RA is transported by a cellular retinoic acid binding protein (CRABP) to the nucleus. CRABP exists in two isoforms, CRABP and CRABPII and both bind to RA but discriminate retinol. It functions by transferring RA into the nucleus for the subsequent reaction. When RA enters into the nucleus, it binds to ligand-activated transcription factors, retinoic acid receptor (RAR). RAR exists in three isoforms (α, β and γ) and is encoded by distinct genes. RA binds to either RARs but only functions in heterodimeric partnership with another group of ligand-activated transcription receptors retinoid X receptor (RXR, α, β and γ). The RA-RAR-RXR complex then interacts with a retinoic acid response element (RARE) in the promoter region of a target gene to activate the transcription of the target gene (Napoli 1999; Bastien and Rochette-Egly 2004). RARE consists of two typical direct repeats of a core hexameric motif, PuG(G/T)TCA (Leid et al. 1992; Mangelsdorf and Evans 1995). Between the two hexameric motifs, there is either 1-bp-, 2-bp- or 5-bp-spaced direct repeat (referred as DR1, DR2 and DR5 respectively) in a complete RARE.

Although RA is being tested as a therapeutic agent in several human cancers, in some tissues, RA is able to promote cell survival rather than cell growth inhibition or cell death (Verma et al. 1982; Henion and Weston 1994;

Jacobs et al. 2006). Recently, Suhug et al. (2007) found that in the cells that RA promoted cell proliferation, other than RARs, RA was able to bind with another class of ligand-activated transcription receptors called PPAPβ/δ, which formed a heterodimer with RXR in RARE to initiate transcription of the target genes. Surprisingly, the RA that bound to PPAPβ/δ was not transferred by CRABP, but by another intracellular lipid binding protein known as fatty-acid binding proteins (FABPs). In HaCaT keratinocyte cells, in response to RA, FABP5, a subclass of FABPs, bound to RA and translocated to the nucleus and then activated PPAPβ/δ transcription. Therefore, it was concluded that RA is able to induce a dual transcription activity either by binding to CRABP or FEBP5, targeting RA to RAR or PPAPβ/δ. If RA is targeted to RAR, this results in cell growth inhibition or apoptosis. Conversely, if RA is transported to PPAPβ/δ, this promotes cell survival and proliferation (Schug et al. 2007).

1.2.4 Apoptosis induced by RA in mammary carcinoma

RA was reported to stimulate cell growth inhibition or apoptosis in many different kinds of carcinoma cells such as mammary carcinoma. In one of the representative publications in this area that appeared in 2005, Donato found that some proapoptotic genes were the targets of RAR and CRABPII in the mammary carcinoma cell line MCF-7. Upon 4 h of treatment with 50 nM RA, several proapoptotic genes including caspase-7 and caspase-9 were up-regulated in MCF-7 cells. In addition, it was observed that RA could directly induce the expression of caspase-9 but not that of caspase-7, and that a consensus RARE sequence was found in the second intron of the caspase-9 gene, indicating that RA was able to induce apoptosis in MCF-7 cells directly by regulating the apoptotic modulators (Donato and Noy 2005). Besides regulating proapoptotic genes, RA also stimulated the modulation of genes involved in cell cycle regulation in MCF-7 cells. Donato et al. (2007) showed that RA was able to induce apoptosis and also cell cycle arrest at G_1 phase in MCF-7 cells by inducing the expression of p53-controlled anti-proliferative gene, B-cell translocation gene, member 2 (Btg2) and the BTG family member Tob1. In addition, Donato et al. (2007) showed that induction of Btg2 did not require *de novo* protein synthesis and there was a consensus RARE in the promoter region of Btg2 gene. These observations thus showed that RA was able to regulate cell cycle arrest and apoptosis by directly stimulating the genes involved in the process.

1.2.5 Apoptosis induced by RA in acute promyelocytic leukemia

RA has already been put in clinical trial for the treatment of acute promyelocytic leukemia disease (Altucci and Gronemeyer 2001). It was shown that RA induced complete remisson in nearly most of the patients with acute promyelocytic leukemia (James et al. 1999). The underlying mechanisms are slightly different from that of mammary carcinoma; and the remissions are the results of RA-induced terminal differentiation of the abnormal promyelocytes, followed by subsequent apoptosis (James et al. 1999). To induce apoptosis in HL-60 acute promyelocytic leukemia cells, Bcl-2 was down-regulated after RA-induced differentiation (Delia et al. 1992; Nagy et al. 1996). Otake et al. (2005) further confirmed this observation and showed that RA-induced apoptosis in HL-60 cells was associated with nucleolin down-regulation and destabilization of Bcl-2 mRNA(Otake et al. 2005), although the mechanism of whether the Bcl-2 gene is directly regulated by RA is still unknown. In addition, Prowse et al. (2002) showed that besides cell terminal differentiation followed by apoptosis, RA could also stimulate cell cycle arrest at G_0/G_1 phase in HL-60 cells by down-regulation of a strong candidate for a tumor suppressor gene OVCA2.

1.2.6 Apoptosis induced by RA in malignant melanoma

RA was also observed to inhibit cell proliferation or induce apoptosis in melanoma cells. Surprisingly, in some of the melanoma cells, only cell growth inhibition but not apoptosis could be induced by RA. One of the examples was sk-mel-28 cells. Emionite et al. (2003) showed that 10 μM RA stimulated cell growth inhibition for 8 days through cell cycle arrest at G_0/G_1 phase. However, the mechanism of cell cycle arrest in sk-mel-28 cells was similar to those found in mammary carcinoma and acute promyelocytic leukemia (G_0/G_1 phase re-distribution). In addition, it was shown that in sk-mel-28 cells, RA stimulated RAR-β up-regulation, indicating that RA interacted with RAR-β and RXR to form a heterodimer complex, and the binding of the complex to RARE would stimulate a series of gene modulation (Emionite et al. 2003).

Although RA did not induce apoptosis in sk-mel-28 cells, it was reported to stimulate apoptosis in primary and matched metastatic cutaneous melanoma cell lines through the mitochondrial pathway. However, the patterns of cell cycle re-distribution of the primary and matched metastatic cutaneous melanoma cell lines were different from that of sk-mel-28 cells when subjected to RA treatment. Primary and matched metastatic cutaneous melanoma cells showed a decrease in the number of cells in G_0/G_1 phase and an increase in G_2 phase, which was totally different from sk-mel-28 cells in which cell cycle was arrested at G_0/G_1 phase. Moreover, primary tumor cells showed a more pronounced sensitivity to RA than that of metastatic cells from the same

patients, suggesting the potential use of RA in early melanoma chemotherapy (Zhang et al. 2003).

Zhang and Rosdahl (2005) found that RA induced apoptosis in the primary and matched metastatic cutaneous melanoma cell lines by alteration of the expression profiles of Id1 and p16 proteins that were important in the regulation of normal cell proliferation and apoptosis. Id1 protein was down-regulated, which resulted in the release of the blocking of p16 proteins, which in turn promoted the increase in the S phase and the G_2 phase of the cell cycle (Zhang and Rosdahl 2005).

1.2.7 Apoptosis induced by RA in hepatocellular carcinoma

RA is also a candidate for the treatment of hepatocellular carcinoma (Okuno et al. 2001; Kojima et al. 2004; Okuno et al. 2004). RA has already been shown to induce cell growth inhibition and apoptosis in hepatocellular carcinoma cell lines such as Hep3B and HepG2. Kim et al. (1996) reported that Hep3B apoptosis was induced by RA with cell cycle arrest at sub G_0 phase. However, RA acted differently in Hep3B and HepG2 cells. Although Hep3B cells were sub G_0 cell cycle-arrested and induced to apoptosis by RA, HepG2 and SNU354 cells were arrested at G_1 phase by RA, and the expressions and activities of CDK2 or CDK4 were decreased (Jung et al. 2005). HepG2 also showed apoptosis when subjected to RA treatment (Arce et al. 2005). In terms of apoptosis, Hep3B and HepG2 cells showed different pathways. Although both cell lines showed DNA fragmentation and apoptosis when RA was added, procaspase-3, a proapoptotic protein, was cleaved into active caspase-3 in Hep3B cells while HepG2 was caspase-independent (Arce et al. 2005).

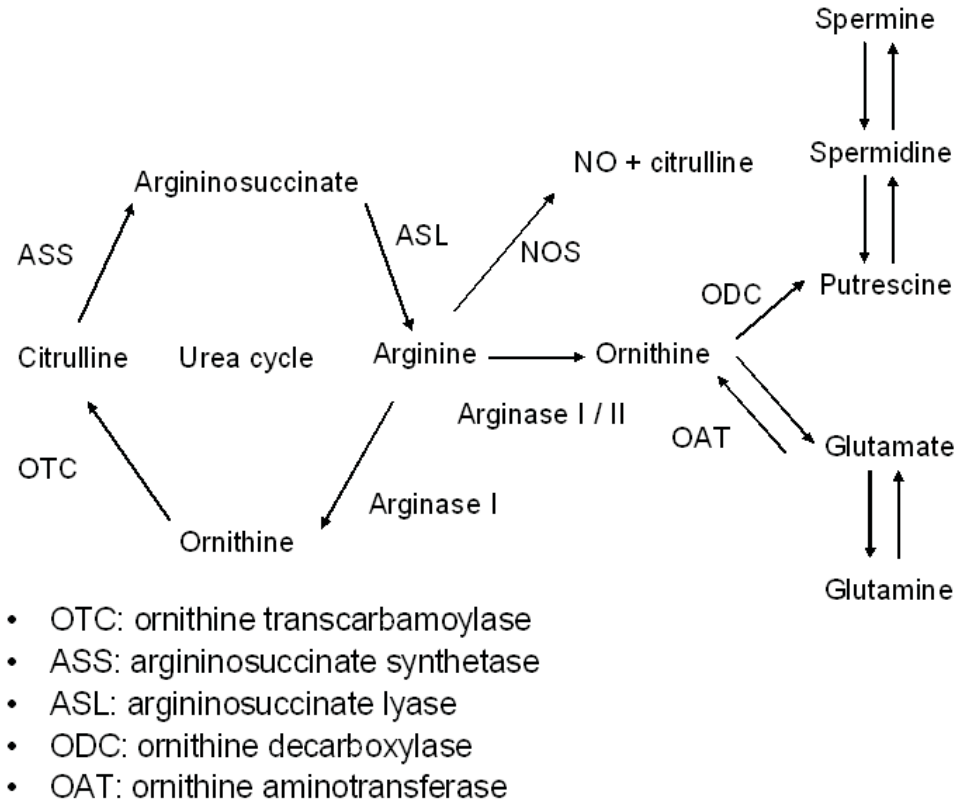
1.3 Relationship between RA and arginase

In 1980, Rhodes and Oliver first discovered that RA at physiological concentrations was able to enhance the production of arginase in normal guinea-pig peritoneal macrophages (Rhodes and Oliver 1980). This was similar to what Currie (1978) had found, that was the elevation of arginase production in activated macrophages by LPS or zymosan killed tumor cells. In addition, Ulland et al. (1997) found that the concomitant administration of RA with corticosterone increased the conversion of arginine into ornithine in wound healing in rat, suggesting that arginase activity was up-regulated by RA. However, the mechanisms and the effects that RA stimulates the induction of arginase are still unknown. Recently, our collaborators found that when RA was injected into pregnant mice for 12 h, the embryos showed caudal regression by apoptosis in tail buds (see supporting information) (Shum et al. 1999). Moreover, it was observed that before the onset of apoptosis, arginase I expression was up-regulated in the tail bud (personal communication with Dr. Shum, see supporting information). Therefore, we hypothesized that RA is able to induce arginase up-regulation directly by stimulating arginase I mRNA expression, but how the increased arginase activity triggers apoptosis in cells is an unanswered question.

1.4 Objectives in the present study

In order to prove that RA is able to induce arginase up-regulation which results in apoptosis in cells, mouse embryonic carcinoma cell line P19 (chosen because of the embryonic and the differentiation property of P19 cells to mimic a similar character of mouse embryo tail bud) and mouse melanoma cell line B16-F0 (chosen because of the cancerous property of B16 cells and for a better understand of the effect of RA on malignant cells) were used in our studies. The two cell lines were studied by *q*RT-PCR and enzyme activity assay to investigate the effect of RA on arginase and by Annexin V-FITC staining technique (flow cytometry) to investigate the effect of RA on arginase-dependent apoptosis. The effects of ornithine and polyamines (putrescine, spermidine and spermine) on both cell lines were also determined by cell viability assay and apoptotic assay. Moreover, P19 cells were used to detect the presence of a consensus RARE sequence in the promoter of the mouse arginase I gene by luciferase activity reporter assay. In addition, cyclic AMP, a popular patented arginase-inducing agent was employed to examine the role of up-regulating arginase *in-vitro* in apoptosis.

To compare the effects between intra-cellular induced arginase and extra-cellular added arginase, bovine arginase was employed to determine if similar effects would be observed. In addition, the cell cycle re-distribution patterns of P19 and B16 cells upon arginase treatment were also investigated by flow cytometry.



- OTC: ornithine transcarbamoylase
- ASS: argininosuccinate synthetase
- ASL: argininosuccinate lyase
- ODC: ornithine decarboxylase
- OAT: ornithine aminotransferase

Fig. 1.1: Schematic representation of the roles of arginase and other arginine-metabolizing enzymes.

2 Materials and methods

2.1 Preparation of chemicals used in cell culture

50 mg RA (Sigma) was dissolved in 16.6 ml absolute ethanol to make a stock of 10 mM. This stock was aliquoted into glass cylinders to prevent any loss of RA. Each aliquot was gassed with nitrogen gas to prevent any oxidation of RA and the stock was kept at -80 °C.

To prepare 100 U/ml bovine arginase (arginase, sigma), 3.05 mg lyophilized stock (164 U/mg) was dissolved in 5 ml PBS. This stock was filter-sterilized by 0.22 μm filter unit and was kept at -20 °C. The stock was used within one month.

5 mg specific arginase inhibitor, N^{ω}-hydroxy-nor-arginine (norNOHA, Cayman), was dissolved in 1 ml dimethyl sulfoxide (DMSO, Sigma) to make a stock of 12.1 mM. This stock was aliquoted into eppendorf with 100 μl each and was gassed with nitrogen gas to prevent any oxidation of norNOHA. The stock was kept at -20 °C.

5 mg arginase inhibitor, N^{G}-Hydroxy-*L*-arginine (NOHA, Calbiochem), was dissolved in 2 ml ddH_2O to make a stock of 10 mM. This stock was filter-sterilized by 0.22 μm filter unit and was gassed with nitrogen gas to prevent any oxidation of NOHA. The stock was kept at -20 °C.

To prepare 5.4 mM putrescine stock, 4.35 mg powder (Sigma) was dissolved in 5 ml ddH_2O. The stock was filter-sterilized by 0.22 μm filter unit

and was kept at -20 °C.

To prepare 6.9 M spermidine stock, 5 g powder (Sigma) was dissolved in 5 ml ddH_2O. The stock was filter-sterilized by 0.22 μm filter unit and was kept at -20 °C.

To prepare 4.94 M spermine stock, 5 g powder (Sigma) was dissolved in 5 ml ddH_2O. The stock was filter-sterilized by 0.22 μm filter unit and was kept at -20 °C.

100 mg N^6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (dibutyryl cyclic AMP/dbcAMP, Sigma) was dissolved in 2.03 ml PBS to make a stock of 100 mM. The stock was filter-sterilized by 0.22 μm filter unit and was kept at -20 °C. All other chemicals were dissolved in PBS and were kept at 4 °C or -20 °C.

2.2 Cell culture

Mouse embryonic carcinoma P19 cells, obtained from ATCC (ATCC number: CRL-1825), were cultured in a 100-mm tissue culture dish (Iwaki) in alpha MEM containing 7.5 % bovine calf serum, 2.5 % fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin (All from Gibco except bovine calf serum which was from ATCC). Mouse melanoma B16-F0 (B16) (ATCC number: CRL-6322) was cultured in a 100-mm tissue culture dish in DMEM containing 10 % FBS, 100 U/ml penicillin and 100 U/ml streptomycin (All from Gibco). All cell lines were cultured at 37 °C in a humidified atmosphere of 5 % CO_2.

When P19 and B16 cells had reached about 90 % confluence (which took about 2-3 days), they were split into 1/10 dilution. To split the cells, the medium was removed from the 100-mm tissue culture dish, and the cells were washed with sterilized PBS. After removing the PBS, cells were subjected to 1 ml of 0.25 % trypsin / EDTA at 37 ^{o}C for 3 minutes for the cells to detach from the culture dish. Then 1 ml of complete medium was added to the culture dish to dilute and inactivate trypsin, and the mixture was transferred to a 50 ml centrifuge tube (BD biosciecnes). The cells were then spun down at 150 g for 3 minutes at room temperature. After removing the supernatant, 1 ml of complete medium was added to re-suspend the cells by repeatedly pipetting, and 100 μl suspension cells were added to a new 100-mm tissue culture dish containing 10 ml complete medium for culture.

For long-term storage of P19 and B16 cells, 10^7 cells were suspended in 1 ml complete medium containing 5 % dimethyl sulfoxide (DMSO, Sigma, U.S.A.) in a 2-ml plastic ampoules (NUNC., Denmark). The cells were stored in an isopropanol-bathed bucket in a -80 °C freezer for 24 h. Then the frozen cell stocks were stored in liquid nitrogen. To check the viability of the frozen cells, one of the cell stocks was thawed rapidly (about 90 sec) in a 37 °C water bath and the cells were added to pre-warmed complete medium at 37 °C for culture. P19 and B16 cells were subcultured and passages below 30 were used in the whole study.

2.3 Cell viability assay

Cells were planted in 96-well tissue culture plates at 5,000 cells in 100 μl of complete growth medium and were allowed to adhere to the plate overnight. On the next day, RA, dbcAMP, arginase, arginase inhibitor, *L*-ornithine, polyamines or in combination at various concentrations were added and cells were allowed to incubate to up to 3 days. For the arginine dependent assay, 5,000 cells were planted in 96-well tissue culture plates in 100 μl of complete growth medium and were allowed to adhere to the plate overnight. On the next day, different concentrations of arginine (0 – 500 μM) were added to the cells in 100 μl custom-made arginine-free medium (USBiological) with 10 % FBS and 100 U/ml penicillin and 100U/ml streptomycin, and incubation was allowed for 3 days. Viability of cells was determined as a function of redox potential by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen) according to the manufacturer's instruction. In brief, treated cells were incubated with 10 μl 5 μg/ml water-soluble MTT reagents in 100 μl culture medium at 37 ^{o}C for 4 h. MTT is chemically reduced by cells into purple formazan, which is then dissolved by acidified SDS (0.01 N HCl in 10 % SDS) in tissue culture medium. Concentration of the cleavage product formazan was then measured by reading its absorbance with a spectrophotometer with a 570 nm filter. Cell proliferation data were expressed as a percentage of control.

2.4 Quantitative reverse transcription-polymerase chain reaction (*q*RT-PCR) and semi-quantitative polymerase chain reaction (RT-PCR)

The *q*RT-PCR technique was used to detect gene expression of arginase I, arginase II, iNOS and beta-actin in mouse cell lines.

RNeasy mini kit (Qiagen) was used to extract total RNA from the cell lines as described in the user's manual. Reverse Transcription was done by using 1 μg total RNA per sample and using RNase H^+ iScript reverse transcriptase of iScript cDNA synthesis kit (Biorad), running at 42 °C for 60 min. The reaction was then incubated at 85 °C for 5 min to inactivate reverse transcriptase. Any remaining RNA was removed by RNase H included in the master reaction mixture. 0.4 μl cDNA sample was amplified by hot-start iTaq DNA polymerase of iQ SYBR green supermix (Biorad) in a 20 μl-reaction mixture. The final reaction mixture was heat-denatured at 94 °C for 3 min, and then subjected to *q*PCR, with amplification for 40 cycles at 94 °C for 30 sec, primer annealing at 61 °C for 30 sec, and extensions at 72 °C for 30 sec. To analyze the specificity of the corresponding sets of primer, melt-curve analysis was performed immediately after the amplification protocol with 1 min denaturation at 95 °C, followed by 1 min annealing at 55 °C and then subjected to 80 cycles of 0.5 °C increments (10 sec each) beginning at 55 °C. The whole PCR took place in an iQ5 real-time PCR detection system (Biorad).

The primers used in the *q*PCR were listed in Table 2.1. 100 ng of sense and antisense primers were used in each 20 μl-reaction.

Arginase I	Sense	5’ GTCTGGCAGTTGGAAGCATCT 3’	69 bp
	Anti-sense	5’ GCATCCACCCAAATGACACA 3’	
Arginase II	Sense	5’ CTGCCATTCGAGAAGCTGG 3’	104 bp
	Anti-sense	5’ GGGATCATCTTGGACATTAG 3’	
iNOS	Sense	5’ ACCATGGAGCATCCCAAGTA 3’	62 bp
	Anti-sense	5’ GGCAGTGCATACCACTTCAA 3’	
Beta-actin	Sense	5’ GGCTGTATTCCCCTCCATCG 3’	153 bp
	Anti-sense	5’ CCAGTTGGTAACAATGCCATGT 3’	

Table 2.1: Primers for *q*RT-PCR.

Semi-quantitative RT-PCR was used to detect the expression of ornithine-consuming enzyme including OTC, ODC and OAT in P19 and B16 cells. After total RNA was extracted by RNeasy mini kit and was converted into first strand cDNA by RNase H^+ iScript reverse transcriptase of iScript cDNA synthesis kit, 0.4 μl cDNA sample was amplified by GoTaq® DNA polymerase (Promega) in 2X GoTaq® green master mix in a 20 μl-reaction mixture. The final reaction mixture was heat-denatured at 94 °C for 3 min, and then subjected to PCR, with amplification for 30 cycles at 94 °C for 1 min, primer annealing at 61 °C for 1.5 min, and extensions at 72 °C for 1.8 min. The primers used in the RT-PCR were listed in Table 2.2. 100 ng of sense and antisense primers were used in each 20 μl-reaction. The PCR products were analyzed by running the product in 0.8 % TBE agarose gel electrophoresis with 100 bp plus DNA ladder (Promega) stained with 0.5 μg/ml ethidium bromide.

Arginase I	Sense	5’ ATGAGCTCCAAGCCAAAGTC 3’	969 bp
	Anti-sense	5’ TCACTTAGGTGGTTTAAGGT 3’	
Arginase II	Sense	5’ ACAGGGTTGCTGTCAGCTCT 3’	298 bp
	Anti-sense	5’ TGATCCAGACAGCCATTTCA 3’	
OTC	Sense	5’ CGTCTTCAAGCTTTCCAAGG 3’	234 bp
	Anti-sense	5’ AAACTTTGGCTTCTGGAGCA 3’	
ODC	Sense	5’ GACGAGTTTGACTGCCACATC 3’	111 bp
	Anti-sense	5’ CGCAACATAGAACGCATCCTT 3’	
OAT	Sense	5’ TGCCACCCAAAGATCATAGATGC 3’	103 bp
	Anti-sense	5’ TGTACTCCTCGTATTCACCAAGG 3’	
Beta-actin	Sense	5’ GGCTGTATTCCCCTCCATCG 3’	153 bp
	Anti-sense	5’ CCAGTTGGTAACAATGCCATGT 3’	

Table 2.2: Primers for RT-PCR.

2.5 Apoptosis assay

Apoptosis of cells was detected by using Annexin V-FITC apoptosis detection kit (BD Pharmingen) according to the manufacturer's instruction. Breifly, 2×10^5 cells were seeded onto a 35-mm dish or 6-well plate. Cells were then grown in complete medium at 37 °C for 24 h. RA, cAMP or arginase at stated concentrations were added to cells. After each time interval, floating cells were collected by centrifugation at 150 g for 3 min while attached cells were harvested by adding 0.25 % trypsin to cells and incubated for 3 min at room temperature to detach the cells. After spinning down both the floating and attached cells and washing with PBS, apoptotic cells were detected by a flow cytometer (FACSAria, Becton Dickson) by double-staining the cells with Annexin V-FITC and propidium iodide (PI) in accordance to the manufacturer's manual. The subsets of cells that were Annexin V-positive, PI-negative (early apoptosis) and Annexin V-positive, PI-positive (Late apoptosis) were determined. About 10,000 cells per sample were acquired in histograms of FASCAria software (Becton Dickinson).

2.6 Cell cycle analysis

Cell cycle analysis was done by labeling fixed cells with propidium iodine (PI) and then analyzed by flow cytometry. 2×10^5 cells were seeded in 6-well plates and incubated overnight. Cells were incubated with different doses of RA or arginase for 24 h. Cultured cells (both detached and attached cells) were then collected by 0.25 % trypsin and centrifuged at 150 g for 3 min, washed twice with PBS, and fixed with 70 % ice-cold ethanol for at least 30 min. After removing ethanol and washing with PBS, cells were stained with PI/RNase staining buffer (BD Pharmingen) for 15 min at room temperature in darkness. Stained cells were analyzed using FACSAria (Beckton Dickinson). The percentages of cells in G_0/G_1, S and G_2/M phases were determined using ModiFit LT^{TM} software (Verity Software House).

2.7 Arginase activity assay

Arginase activity of cell samples was analyzed by using QuantiChrom arginase assay kit (BioAssay Systems) according to the manufacturer's instruction. Briefly, 2×10^5 cells were seeded onto a 35-mm dish or 6-well plate. Cells were then grown in complete medium at 37 °C for 24 h. RA or cAMP was added to cells. After incubation, cells were harvested by 0.25 % trypsin, collected by centrifugation at 150 g for 3 min and washed twice with ice-cold PBS. Pelleted cells were lysed with lysis buffer (Promega) at 4 °C with vigorous shaking for 30 min. Cell lysate was removed by centrifugation at 12,000 g for 10 min at 4 °C. 40 μl supernatant was used for activity assay with 10 μl 5 × substrate buffer (4 volume of arginine buffer and 1 volume of manganese solution). Reaction was incubated at 37 °C for 2 h and the arginase activity was determined by detecting urea formation by incubation with 200 μl urea reagent (reagent A and reagent B) for 15 min at room temperature in dark. Optical density was read at 520 nm. Arginase activity was expressed as the unit of arginase converting 1 μmole of *L*-arginine to *L*-ornithine and urea per minute per mg protein at pH 9.5 and 37 °C.

2.8 Construction of reporter plasmids for RARE analysis

2.8.1 Preparation of 2kb genomic DNA sequence upstream of the mouse arginase I transcription start site

A genomic fragment (located on chromosome 10: A4, 24601297 – 24761536) containing the whole arginase I gene (24635029 – 24647244) including exon and intron was embedded in a bacterial artificial chromosome (BAC), which had been ordered from the Children's Hospital Oakland Research Institute (CHORI) (cat. no. RP23-13L4). The bacterial BAC clones were streaked on a LB agar plate with 12.5 ug/ml chloramphenicol and the plate was then incubated at 37 °C overnight. On the next day, a single colony was isolated from the plate and was inoculated in a 5 ml starter culture containing chloramphenicol, which was incubated at 37 °C for 8 h at 280 rpm. Then 1 ml of the starter culture was diluted into 500 ml LB medium containing chloramphenicol, which was incubated at 37 °C for 16 h at 280 rpm. The bacteria were pelleted by centrifugation, and were then subjected to Large Construct Kit purification according to the manufacturer's description (Qiagen) to isolate the genomic BAC. An approximately 2-kb upstream sequence from transcription start site of the mouse arginase I gene (nucleotides -2000 to 0) was generated by PCR amplification by iProof high-fidelity DNA polymerase (Biorad) (30 cycles of 98 °C 10 sec, 60 °C 30 sec and 72 °C 1 min) using the following primers:

Sense (5’ - CGGGGTACCAATGGACGCAACAGTCAGCA – 3’) and antisense (5’ - CCCAAGCTTTAACCCTAAAAGACAGAGGG – 3’). The purity of the PCR product was revealed by running the product in 0.8 % TBE agarose gel electrophoresis with 1 kb plus DNA ladder (Promega) stained with 0.5 μg/ml ethidium bromide.

2.8.2 Isolation of PCR product

The desired PCR product, after checked by DNA electrophoresis, was extracted by illustra GFX PCR DNA and Gel Band Purification Kit (GE healthcare) according to the manufacturer's instruction.

2.8.3 Restriction enzyme digestion of purified PCR product and plasmid vector

The purified 2 kb genomic DNA fragment containing PCR-generated restriction site and the vector pGL3-basic (kindly provided by Dr. Daniel Lee) were digested separately in a 50 μl mixture containing 1 unit of *Kpn*I (NEB), 1 x reaction buffer and 1 μg 2 kb genomic DNA or pGL3-basic, incubated for 16 h, and then purified by illustra GFX PCR DNA and Gel Band Purification Kit (GE healthcare). The *Kpn*I-digested and purified 2 kb genomic DNA and pGL3-basic plasmid were then digested separately by 1 unit of *Hind*III (NEB) in 1 x reaction buffer in 50 μl mixtures. After incubation for 16 h, the digested DNA was purified and was used for ligation.

2.8.4 Ligation of 2 kb fragment and pGL3-basic to generate pGL3-AI

After digesting the 2 kb genomic fragment and pGL3-basic plasmid with *Kpn*I at 5' and *Hind*III at 3' end, the fragment was isolated from restriction enzyme and was ligated into the pGL3-basic luciferase reporter vector at a *Kpn*I site and a *Hind*III located on the multiple cloning site of the vector by T4 ligase (Roche) to generate the construct pGL3-AI. About 1:3 molecular ratio of vector to insert DNA was used to clone the 2 kb genomic DNA into pGL3-basic vector after the restriction digestion. The ligation reaction mixture contained 1 unit of T4 DNA ligase (Roche), 1 X ligase buffer, insert DNA and the vector, and the final volume was adjusted to 20 µl with ddH_2O. The ligation reaction was incubated at room temperature for 5 min and was then used for transformation.

2.8.5 Preparation of competent bacterial cells

Top 10 *E. coli* strain was used for transformation in the entire experiment. To prepare competent cells of Top 10 *E. coli*, *E. coli* strain was streaked on a LB plate without any antibiotics and was incubated at 37 °C overnight. A single colony was isolated from the incubated plate in 10 ml LB broth without antibiotics, which was then incubated at 37 °C overnight at 280 rpm. On the next day, 2 ml overnight culture was diluted into 40 ml pre-warmed LB broth, and was incubated at 37 °C until the OD_{600} reached 0.5. The bacterial cells were then centrifuged at 4 °C for 10 min at 600 g after the cells were chilled on ice for 20 min. The pellet was re-suspended in 20 ml ice-cold 0.1 M $CaCl_2$ and was then chilled on ice for 20 min. The re-suspended bacterial cells were then centrifuged at 4 °C for 10 min at 600 g. The pellet was re-suspended in 3 ml ice-cold 0.1 M $CaCl_2$ and was chilled on ice for 2 h. After that, 3 ml 50 % glycerol was added and then gently mixed. 50 µl aliquot was used for each transformation or kept at -80 °C for storage.

2.8.6 Transformation of ligated product into competent cells

5 µl ligation product was added into 100 µl competent cells and then they were incubated on ice for 30 min. Then the cells were heat-shocked at 42 °C for exactly 2 min and then put on ice for another 2 min. 500 µl LB broth was added to the cells and then incubated at 37 °C for 1 h at 280 rpm. The cells were then streaked on a LB agar plate containing 100 µg/ml ampicillin, and the plate was incubated at 37 °C overnight for bacterial growth.

2.8.7 Generation of mutant promoter constructs

The mutant promoter constructs were generated by performing a site-directed mutagenesis, using iProof high-fidelity DNA polymerase (Biorad), to selectively mutate the possible RARE sequences. The following primers were used for the site-directed mutagenesis: for generation of pGL3-AI mut RARE1: sense (5' – CTTCTTTTCTTTTTACCTTTGGGGCAGGTTCTGTGTGCTCCTTACTACT AACCCTATAACCCCAG – 3') and antisense (5' – CTGGGGTTATAGGGTTAGTAGTAAGGAGCACACAGAACCTGCCCCAA AGGTAAAAAGAAAAGAAG – 3'); for generation of pGL3-AI mut RARE2: sense (5' – ACCAAATGGGTTCTTCGGGAGTAACTCGGGAGCTCGGGCTTGGTGTT ACCGG -3') and antisense (5' – CCGGTAACACCAAGCCCGAGCTCCCGAGTTACTCCCGAAGAACCCAT TTGGT -3'). The PCR profile for the site-directed mutagenesis was as follow: 1 cycle of 95 °C 30 sec, then 18 cycles of 95 °C 30 sec, 55 °C 1 min and 68 °C 2 min. After PCR amplification, 1 unit of *DpnI* was added into the PCR mixture to digest the original DNA template at 37 °C for 4 h. All the constructs used for transfection experiment were confirmed by DNA sequencing and were purified by silica gel membrane (Qiagen).

2.8.8 Transient transfection into P19 cells

Transfection was performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's description. Briefly, 8 X 10^4 cells were seeded on each well of a 24-well plate (Iwaki) in complete alpha MEM without any antibiotics and were allowed to incubate overnight. On the next day, cells were transfected with 0.4 μg DNA using 1 μl lipofectamine 2000. After cells were incubated for 5 h, medium was replaced with complete growth medium and cells were then incubated for another 24 h, followed by the addition of different concentrations of RA. Cells were then incubated for another 24 h until they were harvested for luciferase reporter assay.

2.8.9 Luciferase reporter assay

Transiently transfected P19 cells were lyzed in 100 µl Glo lysis buffer (Promega) for 5 min for lysis to occur at room temperature. Lysates were then transferred to a 1.7-ml eppendorf tube and an equal volume of Bright-Glo Assay Reagent (Promega) was added to it, mixed thoroughly and luminescence was immediately measured by a GloMax^{TM} 20/20 luminometer (Promega) using the default 'Bright-Glo' protocol by the manufacturer.

2.9 Protein measurement

Protein was measured using Bradford assay (Biorad). Briefly, 2 µl protein lysate was added in 1 ml assay mixture containing 200 µl Bradford reagent and 798 µl ddH_2O. Samples were mixed and incubated for 10 min. Protein content was measured at 595 nm. Protein concentration was determined by using a standard protein of bovine serum albumin performed in parallel with the tested samples and was expressed as µg/µl.

2.10 Statistical analyses

For all statistical analyses, results are presented as means ± standard error of the mean (SEM). Further analysis was performed using either two tailed *t*-tests or one way analysis of variance (ANOVA) with Dunnett's multiple comparison test with $p < 0.05$ considered as significantly different.

A

5’ -2000

AATGGACGCAACAGTCAGCAATCACTGTGAATAGCAAATTCTTCATGCATCC
AAGACTTAAGCCCAGCATCTTTCAGGCAGGAAAACAATCTACCCCTGAATGGCATTT
TTCTTCTTTTCTTTTTACCTTTGGGGCAGGTTCTGTGT**TGACCTTTGAACT**AACCCTA
TAACCCCAGCAGGTGTTGAACCTATAATCCCCCTGCCTCATCCTCTCAAATAGCTGG
AATTTGTGGGCCAGCACCAAGAGGCCCAAAGAAAGAGAAAGTTTAAATTAAATAGA
AGAAAATGTAGAGGTATTGACAGGTGGTGGTATCACACACCTTTAATCCCAGCACTC
AGGAGGCAGTGACAGATTGCTATCTGTGAGTTCAAGGTTAGCCTGGTCTATAGGGCT
AGTTCTAGGGTAGCCAGAGCCACACAAAGAAAACATGTCTCCAAAAAGCAAACAA
AAATGTAGAGGTGCACATTTGGGATCTGGGAGCAGGTAAAACTTAAATTCCTAGCTA
GCTTCAAATATATAGGAAAATTCTATCTTACATAAATCAATTTTTTAAAAAAATCTATA
ACCGTATATCAGAAAAACATAGTAAGATGAAAAATATATTTGAAAAGGAGGTATCAA
AATGGTCCTGAGGGACTTTGGCTTCTTGATTTATTCATTCAATAAATGCAATACATTTA
TTCAATCAATAAGTCAATGTTTATTTTGTACTGAACGATTCTATGTACTGATGGTAGAG
GGAACAGAATCTTTCTTGAGGTATTAAAATTCTGGGTGCTTTTTGTCCTCACAAGGTT
AAAGAAATTTACTTCTGACATGTGGGTTTTCTTTTTAAAATTATAATCATGTAACTTTG
TAACTGGGAGAATACTTGGTTGTGTTTCTTCTAAATTAATACTTTTAAAAATATTTCTT
TAGTGTTGGGGGTGGCTTTACACAGGGACCGGACCACATCACCCACGTGGAGGTCA
GAGATCAACTTGGAGGAGTTGACTCTTTCCTTGCACCAAATGGGTTCTTC**GGGTCA**
AACTCGGGTCATCGGGCTTGGTGTTACCGGCTGGCCTCTCTCATCTGCCCTAGATCT
CAGGGGGCCCTGCTGACACTCTGCTGGTGTGTAGATTAGAATCGCGGCAGCTCGGC
GGGTGCAGAATGCCCATTGCTCCGTTTCGATTCTTCTGCAACCTGTATGTGACTAGC
AACCTCACCAAGCTGCAGTCTCTTCCTGGGCAAAACAGCATAAGAATGATTCTCAGT
CTGAAGTTAGTTTAAAGAGGAAACAGGAAGCGAAAGAACATTAACTAGAATAGCAC
TTGGCACACGACAAAGACAACTCATATTAGCTAGTTTGCTTTCCCTATCTTGGGATAT
CATGATTCCAAAAATGAGATTTTCCTGGAGACTGTATTTCAAAAATAGAACTGCTTTG
GGTTGTCAGGGAAATAAATGATGCCTTCCTGTAAATAATAATAATAATAACAACAACA
ACAATATAAAACAAAAACCAGCCCCATGCTTTCCTAGACAGTGTAACCTGGTGACAC
ATGAAATGTGTCTCACTTTCCCCAGAACTTGAAGCCTTGACTCAGGATGCTCAACAG
GAGGGAAGTAAGAGACACCCCCCCCACCCCCTCACCCCCCCCCCCCCACTTGCTGTT
TTAGCCTCACCTTGCAAGTCCCCAGTGGACTTAAATCCTGGAAAAGGTGAGCACCC
TGCCCTGAGGTGCCCAGGCCGGAAGCCTAGCACTTCACATGAGGTTATGAAATCAC
ACATAATTGTCAATTGTCTGAGGAGAGATTAATGTCATCCAGCTGGCTTTTTCAAAAG
GGTGTGAACTGGACGGATGAATAATGCTCAGAGGGAAGAATGGTAGTTCCTCTGAT
GGGGAGGTTCTGTTGACTCTGTCATTCTTCCATTCGGTGGGCGGAGCCAGTTGTTGG
ATAAACAGATCCAACCTGATTATAAGGGGGGAAAAAGATGTGCCCTCTGTCTTTTAG
GGTTA 3’

-1

B

pGL3-AI (RARE-WT) (-1853 – -1841)	5’ – GT**TGACCTTTGAACT**AA – 3’
pGL3-AI mut RARE1 (mutation 1)	5’ – GT**GCTCCTTACTACT**AA – 3’
pGL3-AI (RARE-WT) (-978 – -962)	5’ – TC**GGGTCA**AACTC**GGGTCA**TC – 3’
pGL3-AI mut RARE2 (mutation 2)	5’ – TC**GGGAGT**AACTC**GGGAGC**TC – 3’

C

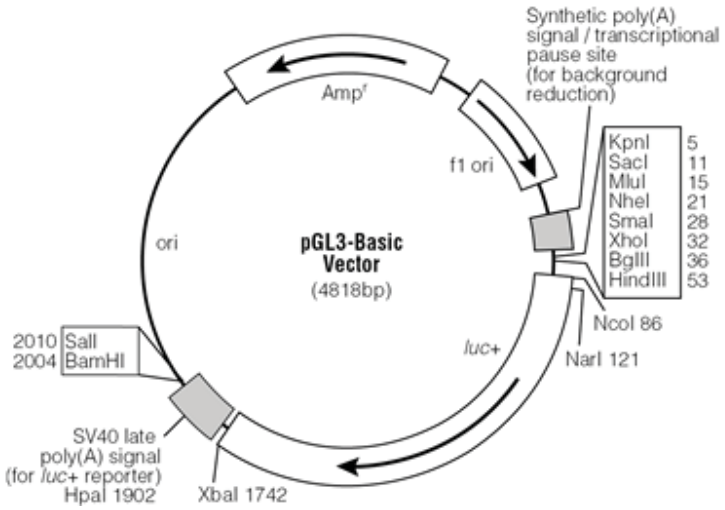


Fig. 2.1: A. 2 kb genomic DNA sequence upstream of mouse Arginase I transcription start site. Bold indicates the two possible RAREs, and underline indicates the direct repeat (DR). The possible RARE1 (-1852 nt – -1843 nt) was found in antisense style while the possible RARE2 (-974 nt – -961 nt) was found in sense. B. The direct repeats of the two possible RAREs were shown in bold, and the mutations of the direct repeats were underlined. C. The pGL3-basic luciferase reporter vector used in the experiments (Adapted from pGL3 luciferase reporter vectors technical manual).

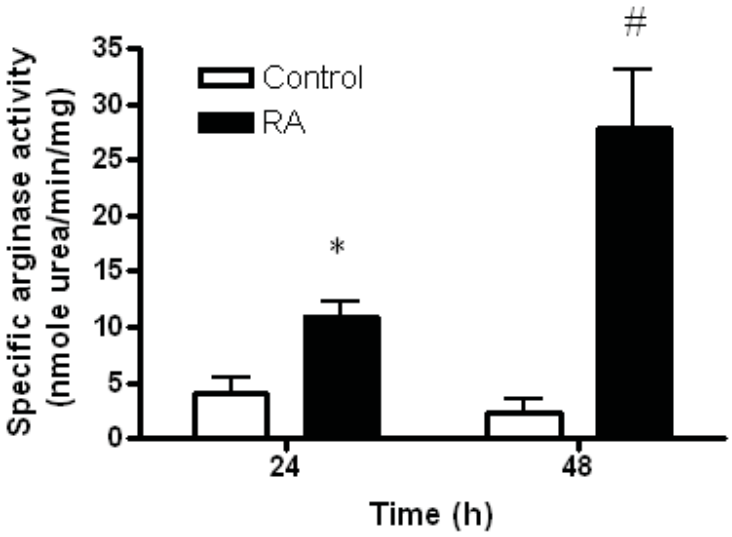
3 Results

3.1 Induction of arginase and apoptosis by *all-trans* retinoic acid in P19 cells

3.1.1 RA stimulates arginase activity by up-regulation of arginase I gene in P19 cells

After 24 h or 48 h of incubation with 10 μM RA, arginase activities were determined in P19 cells by arginase activity assay. As shown in Fig. 3.1A, arginase activity was significantly increased by 2.2-fold and 11.8-fold at 24 and 48 h, respectively. To determine the contribution of the two arginase isoforms to the increased arginase activity, we assessed the levels of mRNA expression of arginase I and arginase II by *q*RT-PCR. Arginase I was up-regulated by 7-fold and arginase II was up-regulated by 1.8-fold after 24 h (Fig. 3.1B).

A



B

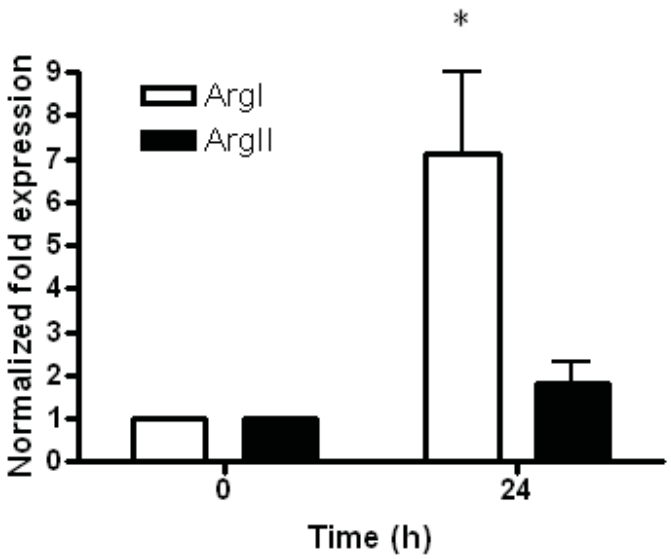


Fig. 3.1: Effect of 10 μM RA on arginase in P19 cells. (*A*), Arginase activity from P19 cells upon RA treatment. P19 cells were subjected to 10 μM RA or 0.1 % absolute ethanol (control). After 24 h, cells were lyzed with lysis buffer,

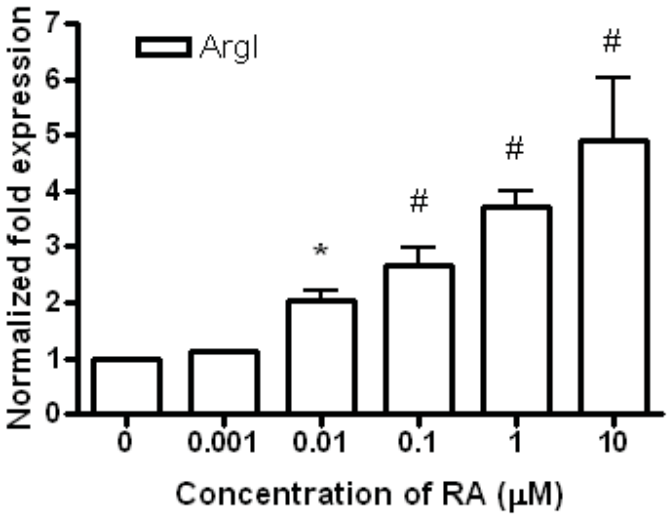
pelleted and supernatant was collected for arginase activity assay. *, $p < 0.05$ (vs control 24 h), #, $p < 0.01$ (vs control 48 h). (*B*), *q*RT-PCR of arginase gene expression. Control and treated cells were harvested, and 0.4 μl first strand cDNA was used for 20 μl *q*RT-PCR. Arginase I (ArgI) and arginase II (ArgII) mRNA expression level were normalized with beta-actin mRNA expression. *, $p < 0.05$ (vs 0 h). Data are the mean ± SEM of three experiments performed in triplicate.

3.1.2 RA induces arginase I gene in dose- and time-dependent manner

To find out the effects of different doses of RA on the arginase I mRNA expression in P19 cells, 0.001, 0.01, 0.1, 1 and 10 μM RA were respectively added to cell culture medium of P19 cells and the cells were then incubated for 24 h before the *q*RT-PCR assay. The results show a higher mRNA expression of arginase I at 0.01, 0.1, 1 and 10 μM RA groups than that in control cells, and the increase of arginase I expression induced by RA was dose-dependent (Fig. 3.2A). Remarkably, 10 μM RA induced the highest expression of arginase I mRNA, which was up to 5-fold compared with the control.

Furthermore, to determine if the effect of RA on arginase I mRNA expression is time dependent, 10 μM RA was added to P19 cells and then incubated for 6, 12, 18, 24 and 48 h, respectively, until the cells were harvested for *q*RT-PCR. The results showed a time-dependent increase of arginase I from 12 h onwards. The expression of arginase I, with an 11-fold increase, was the highest at 48 h. This was consistent to the results of the arginase activity assay. In addition, arginase II mRNA expression was assessed by *q*RT-PCR, and it was observed that arginase II mRNA was up-regulated only at 18 and 24 h by 1.8-fold. However, the RA-stimulated increase in arginase II mRNA expression was not significantly different from the control at time 0 (Fig.3.2B).

A



B

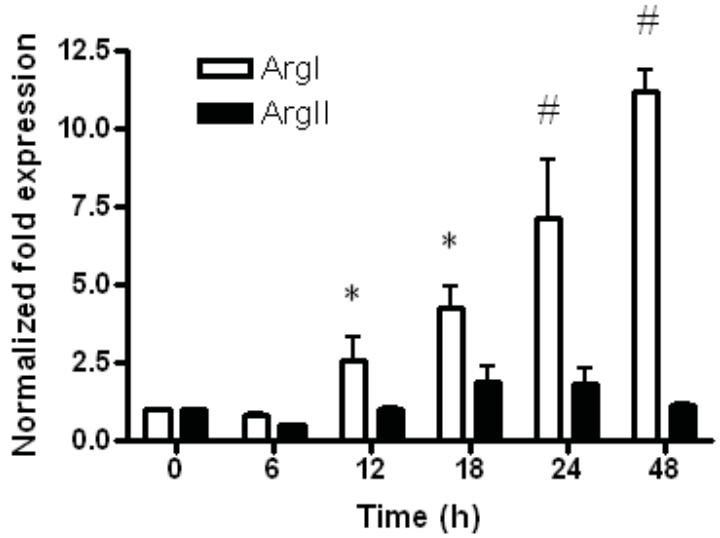


Fig. 3.2: RA increases ArgI mRNA expression in a dose- and time-dependent manner. (*A*), Different concentrations of RA were incubated with P19 cells for 24 h and mRNA level was determined by *q*RT-PCR. *, $p < 0.05$; #, $p < 0.01$ (vs 0 μM). (*B*), P19 cells were incubated with 10 μM RA for the durations

indicated and mRNA level was determined by *q*RT-PCR. *, $p < 0.05$; #, $p < 0.01$ (vs 0 h). Arginase I and arginase II mRNA expression levels were normalized with beta-actin mRNA expression (*A* & *B*). Data are the mean ± SEM of three experiments performed in duplicate.

3.1.3 RA does not stimulate iNOS up-regulation in P19 cells

Since arginine is a common substrate for arginase and nitric oxide synthase (NOS), it is interesting to assess whether NOS is also regulated by the addition of RA in P19 cells. mRNA expression levels of inducible NOS (iNOS) were examined by the *q*RT-PCR assay after 10 μM RA was added to P19 cells for 24 h. There was a slight 1.16-fold stimulation in the 10 μM RA group and there were no significant differences in the expression level of iNOS mRNA between the control group and the 10 μM RA treatment group (Fig. 3.3).

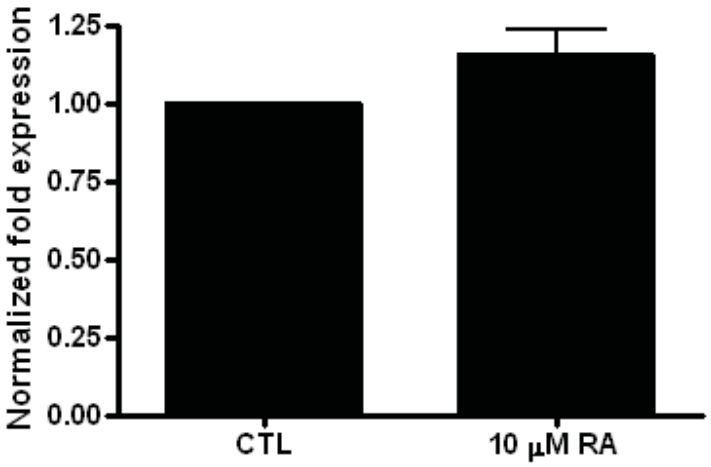


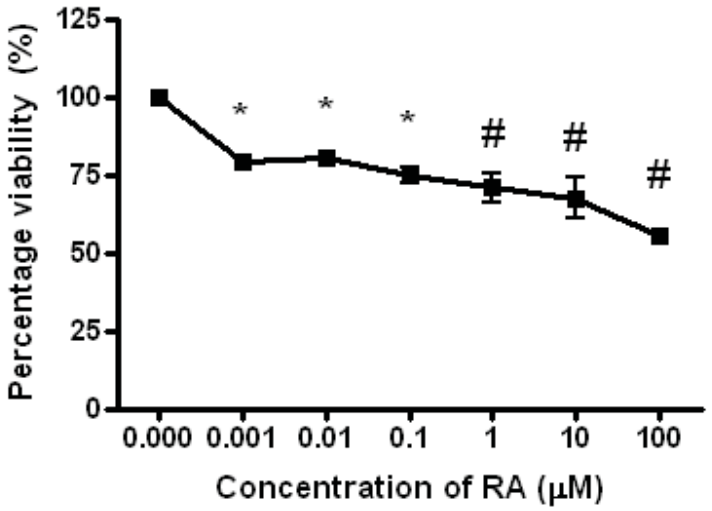
Fig. 3.3: RA did not induce any up-regulation of iNOS in P19 cells. Cells were subjected to 10 μM RA treatment for 24 h and then harvested. After total RNA extraction, 1 μg total RNA was converted into cDNA, and 0.4 μl first strand cDNA was used for 20 μl *q*RT-PCR. iNOS mRNA expression was normalized with beta-actin. There were no significant differences between the control and the 10 μM RA group on iNOS mRNA expression levels ($p > 0.05$). Data are the mean ± SEM of three experiments performed in duplicate.

3.1.4 RA induces P19 cell death by apoptosis

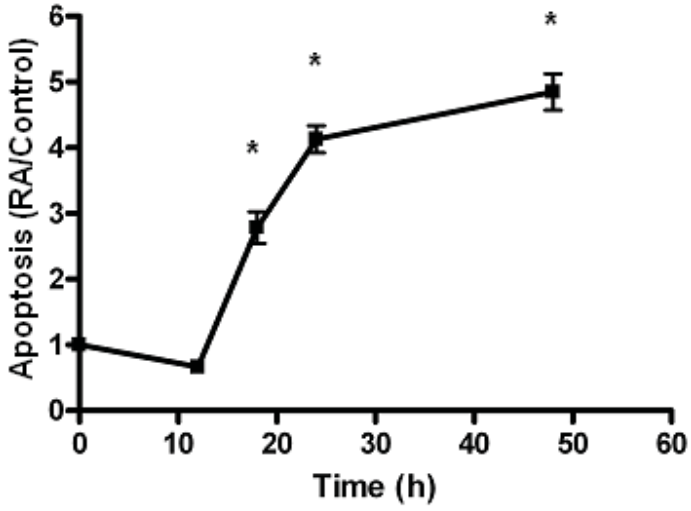
To investigate whether RA affects the growth of P19 cells, a cell viability experiment was done by performing MTT assay. In Fig. 3.4A, when different concentrations of RA were added to P19 cells for 24 h, a dose-dependent cell growth inhibition was observed from 0.001 μM to 10 μM RA. At the lowest tested concentration of RA (0.001 μM), ~ 21 % of P19 cells were growth-inhibited, while at the highest tested concentration of RA (10 μM), ~ 32 % of cells were growth-inhibited.

Next, effect of RA on apoptotic cell death was examined in P19 cells. P19 cells were grown in the presence of 10 μM RA for 12, 18, 24 or 48 h until being harvested for apoptosis detection by Annexin V-FITC staining. Apoptosis was not increased before 12 h, but was significantly increased by 2.67-, 4- and 4.32-fold, at 18, 24 and 48 h, respectively (Figs 3.4B and 3.4C).

A


Percentage viability (%)
125
100
75
50
25
0
*
*
*
#
#
#
0.000
0.001
0.01
0.1
1
10
100
Concentration of RA (μM)

B


Apoptosis (RA/Control)
6
5
4
3
2
1
0
*
*
*
0
10
20
30
40
50
60
Time (h)

C

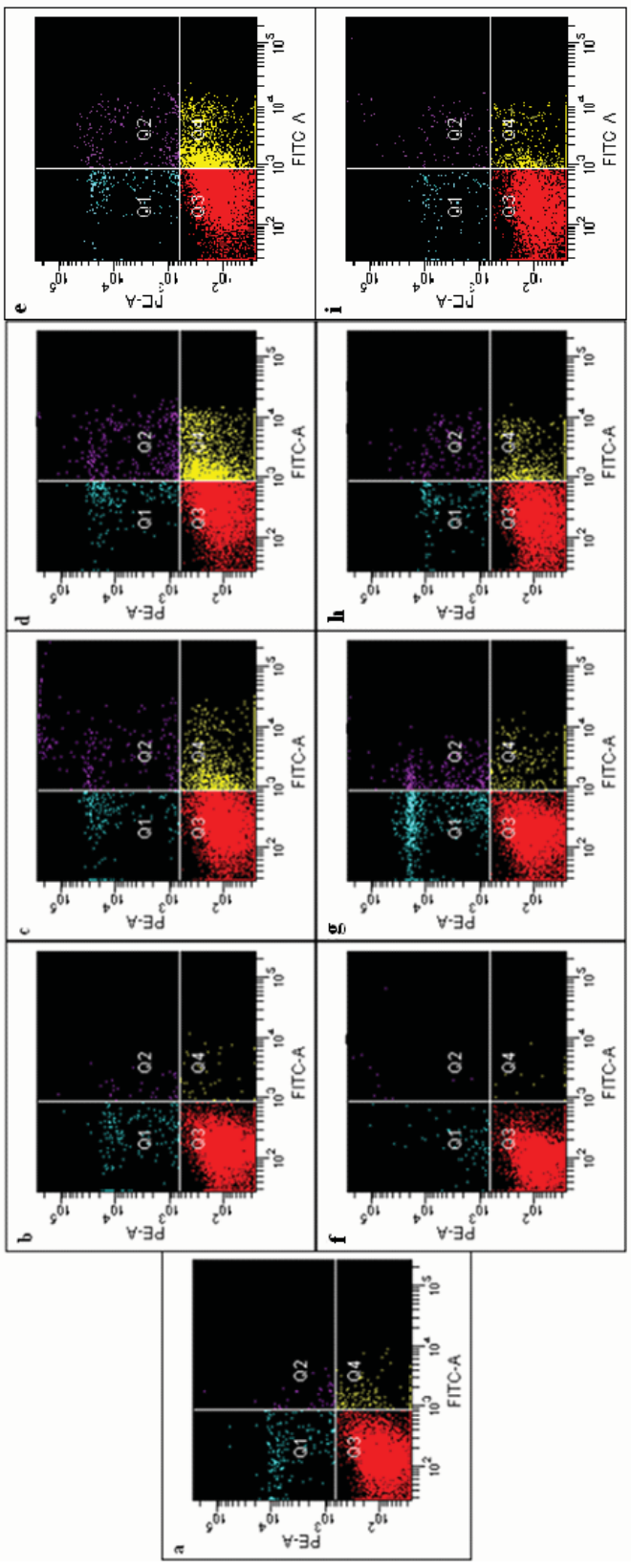


Fig. 3.4: RA-treated P19 cells were studied by MTT assay and flow cytometry using the Annexin V-FITC staining kit. (*A*) MTT assay of RA-treated P19 cells. Different concentrations of RA (0 – 100 μM) were used to incubate the cells for 24 h. *, $p < 0.05$; #, $p < 0.01$ (vs 0 μM). Data are expressed as percentage of control treatment and are the mean ± SEM of three experiments performed in triplicate. (*B*) Apoptosis levels of RA-treated cells (10 μM) were normalized with cells in control experiment. *, $p < 0.05$ (vs 0 h). Data are the mean ± SEM of two experiments performed in triplicate. (*C*), Representative data from triplicate experiments. Q1: Necrosis; Q2: Late apoptosis; Q3: living cells; and Q4: Early apoptosis. a, f, g, h & i: control; b, c, d & e: 10 μM RA; a: 0 h; b & f: 12 h; c & g: 18 h; d & h: 24 h; e & i: 48 h.

3.1.5 RA-induced apoptosis in P19 cells correlates with increased arginase I expression

RA stimulated arginase I up-regulation in P19 cells. Also, apoptosis was observed when RA was added to P19 cells. The question we asked was “Are the two events correlated?” RA-induced arginase I up-regulation occurred after 12 h of incubation and the level peaked at 48 h. However, RA did not stimulate any apoptosis after 12 h of incubation. The RA-induced apoptosis (2.67-fold increase) in P19 cells occurred after 18 h of incubation, at which arginase I was already up-regulated by 4.24-fold. Therefore, RA might induce arginase I up-regulation which in turn resulted in apoptosis in P19 cells.

3.1.6 RA-induced apoptosis is arginase-dependent in P19 cells

Because arginase I expression was up-regulated before the onset of apoptosis induced by RA, the effects of the specific arginase inhibitor, norNOHA, on RA-induced apoptosis was examined in P19 cells. Two doses of RA, 1 μM and 10 μM, in the presence or absence of 100 μM norNOHA, were tested for 24 h. Comparing with the control, apoptosis was significantly increased by 2.42- and 4.23-fold with 1 μM and 10 μM RA, respectively. However, in the presence of norNOHA, apoptosis was reduced to 1.66- and 2.53-fold with 1 μM and 10 μM RA, respectively. The combination of norNOHA and RA significantly lowered the apoptosis in P19 cells when comparing with that induced by RA alone. No change of the basal level of apoptosis in control cells was observed treated with norNOHA alone (Fig. 3.5).

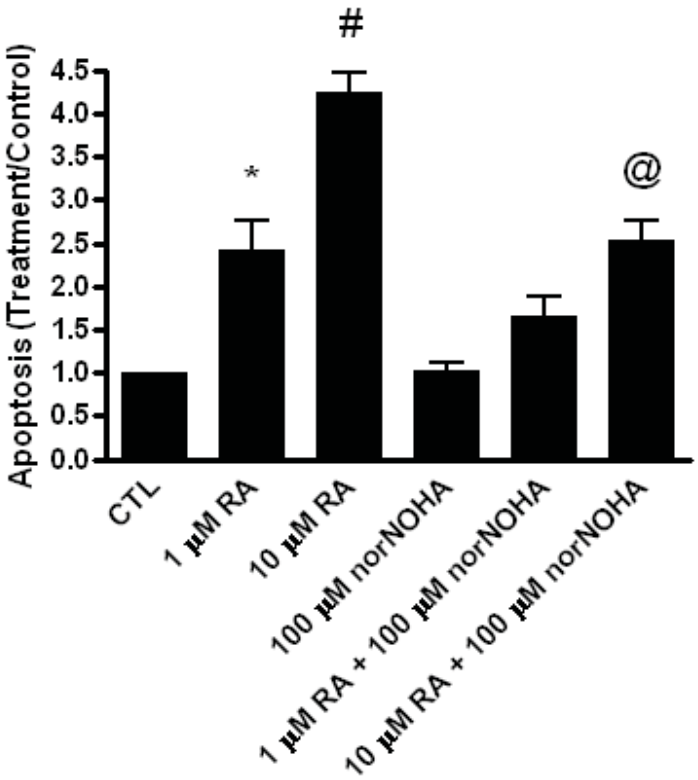


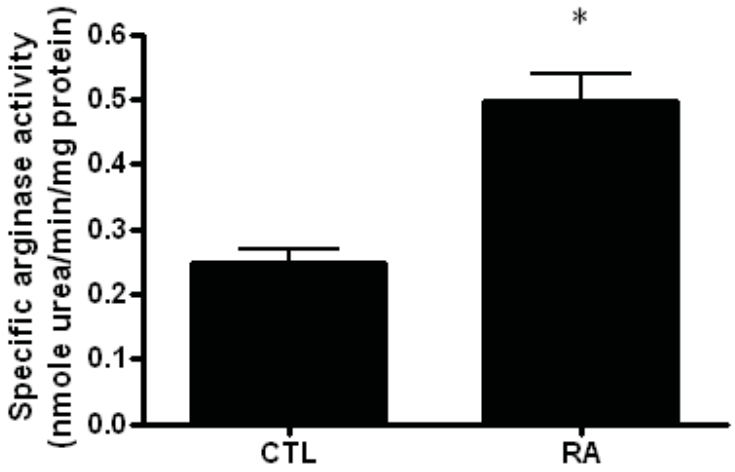
Fig. 3.5: Arginase regulates RA-mediated apoptosis. P19 cells were incubated for 24 h with different concentrations of RA in the presence or absence of norNOHA (100 μM). Apoptosis levels were assessed by flow cytometry (Annexin V-FITC, PI staining). Values are expressed as the mean ± SEM of three experiments performed in duplicate. *, $p < 0.05$; #, $p < 0.01$ (vs control); @, $p < 0.01$ (vs 10 μM RA).

3.2 Induction of arginase and cell growth inhibition by RA in B16 cells

3.2.1 RA induces arginase I expression in B16 cells

B16 melanoma cells were exposed to 10 μM RA for 24, 48 or 72 h and then subjected to arginase activity assay and *q*RT-PCR assay. At 24 h there was a significant 2-fold increase in arginase activity (Fig. 3.6A). To determine the relationship between mRNA and protein expression levels of arginase after RA treatment for 24, 48 or 72 h, *q*RT-PCR experiments of the two arginase genes, (arginase I and arginase II) were performed and the two gene expression levels were normalized with the beta-actin gene control. The mRNA expression level of arginase I gradually increased with time. At 24, 48 and 72 h, there were 1.41-fold, 4.88-fold and 7.84-fold increase in mRNA levels, respectively. There was also a gradual increase in arginase II mRNA expression. Although there was a slight increase in arginase II mRNA expression at 24, 48 and 72 h (1.82, 2.20 and 3.31-fold, respectively), it was not significant when compared with arginase I mRNA (Fig. 3.6B). Therefore, the increased arginase activity upon RA treatment in B16 cells was mainly correlated to the up-regulation of the arginase I mRNA expression.

A



B

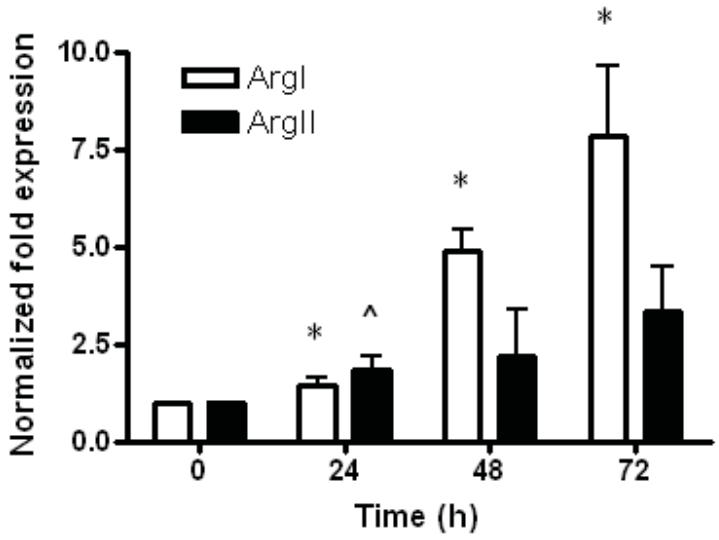


Fig. 3.6: Effect of 10 μM RA on arginase expression in B16 cells. (*A*), Arginase activity in B16 cells upon RA treatment. B16 cells were treated with 10 μM RA

or 0.1 % absolute ethanol (control). After 24 h, cells were lyzed and supernatant was collected for arginase activity assay. *, $p < 0.05$ (vs control). (*B*), *q*RT-PCR studies of arginase gene expression levels up to 72 h. Control and treated cells were harvested, and 0.4 µl first strand cDNA was used for 20 µl *q*RT-PCR. Arginase I and arginase II mRNA expression levels were normalized with the beta-actin mRNA expression. *, $p < 0.05$ (vs ArgI 0 h); ^, $p < 0.05$ (vs ArgII 0 h). Data are the mean ± SEM of three experiments performed in triplicate.

3.2.2 RA stimulates iNOS up-regulation in B16 cells

RA induced arginase up-regulation in both mRNA and protein levels, which means that more arginine would be hydrolyzed by the up-regulated arginase. Arginine is a common substrate for both arginase and NOS. The up-regulation of arginase may affect the expression of NOS which competes for arginine with arginase. Therefore, the expression of NOS mRNA was determined by *q*RT-PCR after 10 μM RA had been added to B16 cells for 24 h. Fig. 3.7 shows that iNOS was significantly up-regulated (2.67-fold) by RA.

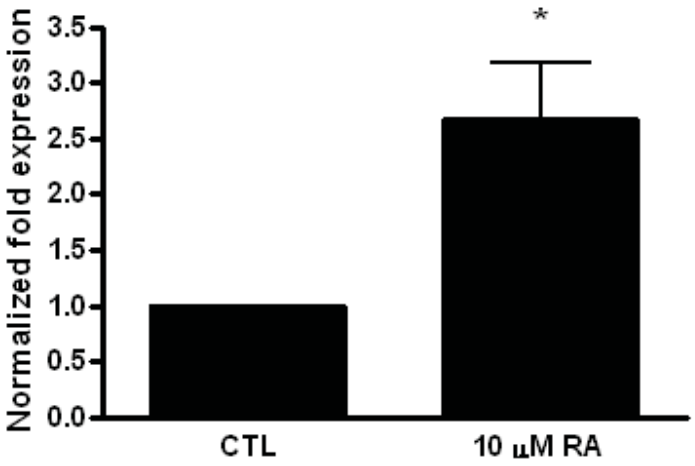


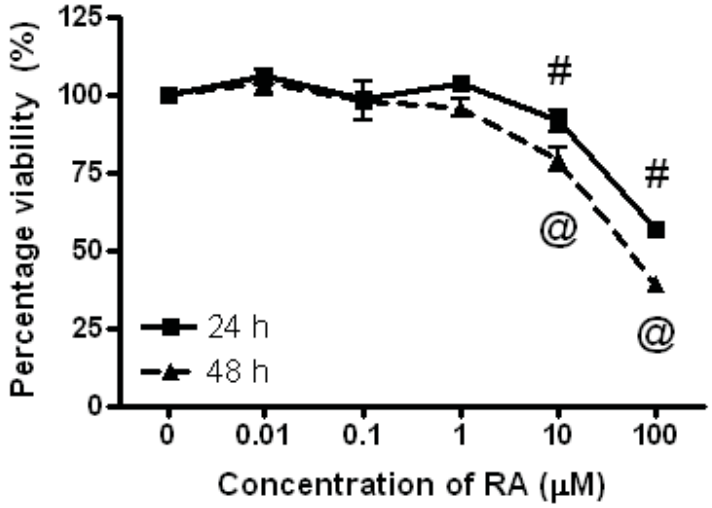
Fig. 3.7: RA induced up-regulation of iNOS in B16 cells upon RA treatment. After 10 μM RA treatment for 24 h, B16 cells were harvested and total RNA was extracted. 1 μg total RNA was converted into cDNA, and 0.4 μl cDNA was used in a 20 μl *q*RT-PCR assay. iNOS mRNA expression was normalized with beta-actin. Data are the mean ± SEM of three experiments performed in duplicate. *, $p < 0.05$ (vs control).

3.2.3 RA induces cell growth inhibition but not apoptosis in B16 cells

To determine the effect of RA on B16 cell proliferation and cell death, cells were exposed to different concentrations of RA for 24 or 48 h and then subjected to MTT cell viability assay. The assay showed that cell growth of B16 cells was significantly inhibited by 10 μM or 100 μM RA at both 24 and 48 h (Fig. 3.8A). Moreover, the pattern of growth inhibition of B16 cells paralleled the expression of arginase I mRNA upon RA treatment, at which arginase I up-regulation started at 24 h, continued at 48 h and peaked at 72 h while growth inhibition of B16 cells was observed at 24 h and continued at 48 h.

RA induced apoptosis in P19 cells after 18 h of incubation. Surprisingly, RA did not stimulate apoptosis in B16 cells even after 72 h of incubation. In the experiment, B16 cells were subjected to 10 μM RA for 24, 48 or 72 h and the cells were harvested for Annexin V-FITC staining to detect apoptosis by flow cytometry. No significant apoptosis induction was observed when compared with the control group (Fig. 3.8B).

A


Percentage viability (%)
125
100
75
50
25
0
24 h
48 h
#
#
@
@
0
0.01
0.1
1
10
100
Concentration of RA (μM)

B

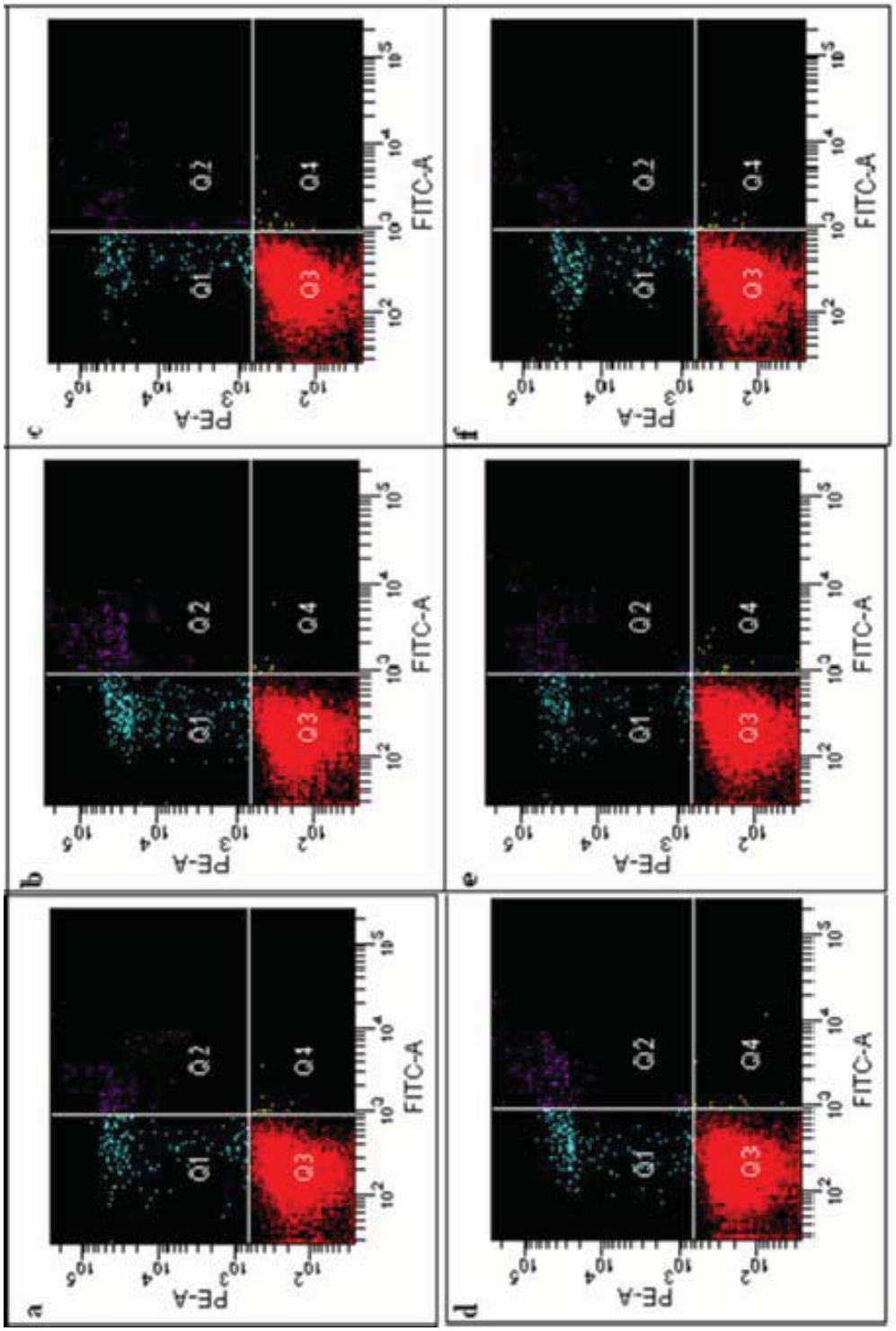


Fig. 3.8: RA inhibits proliferation of B16 cells. (*A*), B16 cells were cultured with 0.1 % absolute ethanol or 10 μM RA in 96-well plates for 24 or 48 h and MTT assay was performed. #, $p < 0.01$ (vs 24 h control); @, $p < 0.01$ (vs 48 h control). Values are expressed as the mean ± SEM of three experiments performed in triplicate. (*B*), Representative data from duplicate experiments. Q1: Necrosis; Q2: Late apoptosis; Q3: living cells; and Q4: Early apoptosis. a, b & c: control; d, e & f: 10 μM RA; a & d: 24 h; b & e: 48 h; c & f: 72 h.

3.2.4 RA-induced B16 cell growth inhibition is arginase-dependent

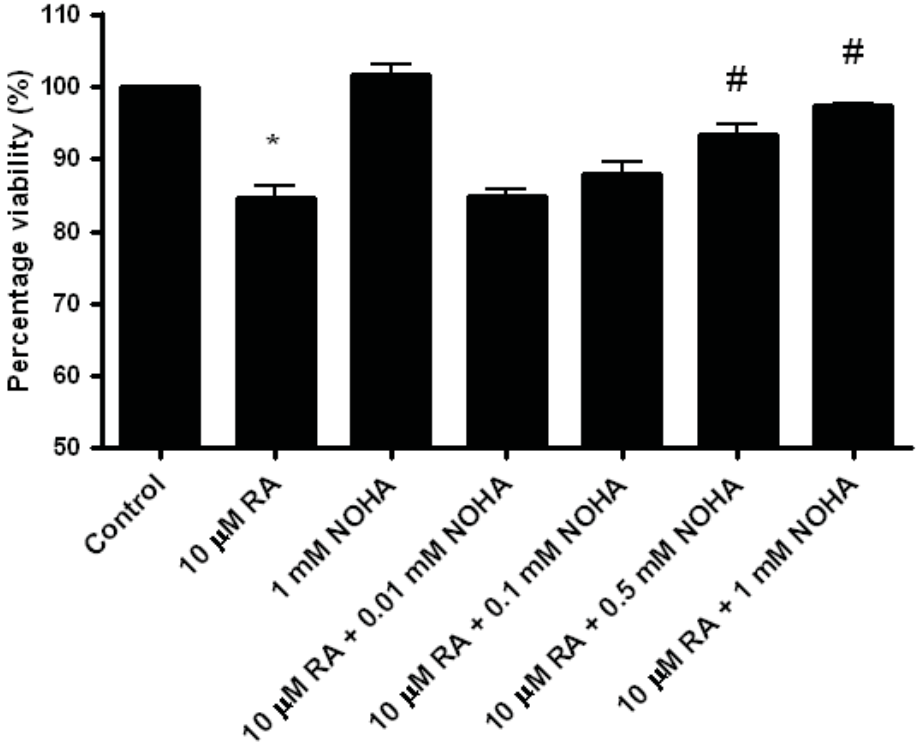
Since arginase activity and ArgI mRNA expression level with increased and paralleled cell growth inhibiton, the effects of the arginase inhibitor N^{ω}-hydroxy-*L*-arginine (NOHA) on the RA-induced cell growth inhibition in B16 cells was assessed to determine whether arginase activity is directly involved. Two approaches were used in the experiments. In the first approach, a fixed amount of RA (10 μM) was exposed to B16 cells together with different amounts of NOHA and after 24 h cell growth was investigated by MTT assay. While the addition of RA alone gave 17 % cell growth inhibition, the addition of different concentrations of NOHA together with RA significantly recovered B16 cells from cell growth inhibition. Adding 1 mM NOHA rescued B16 cells fully from RA-induced cell growth inhibition (Fig. 3.9A). NOHA itself did not stimulate any cell growth inhibition in B16 cells.

In the second approach, a fixed amount of NOHA at 1 mM plus different doses of RA from 0.01 μM to 100 μM was added and after 24 and 48 h, cell proliferation was determined by MTT assay. There were no significant changes in cell growth inhibition between 0.01 μM RA and 0.01 μM RA plus 1 mM NOHA for 24 and 48 h. However, when the cells were exposed to higher doses of RA (0.1 μM, 1 μM and 10 μM; 24 and 48 h) in addition of 1 mM NOHA, cell proliferation resumed when compared to treatment with RA alone. Surprisingly, when cells were exposed to 100 μM RA and 1 mM NOHA, cell proliferation could not fully resume but only recovered from 57 % to 72 % and

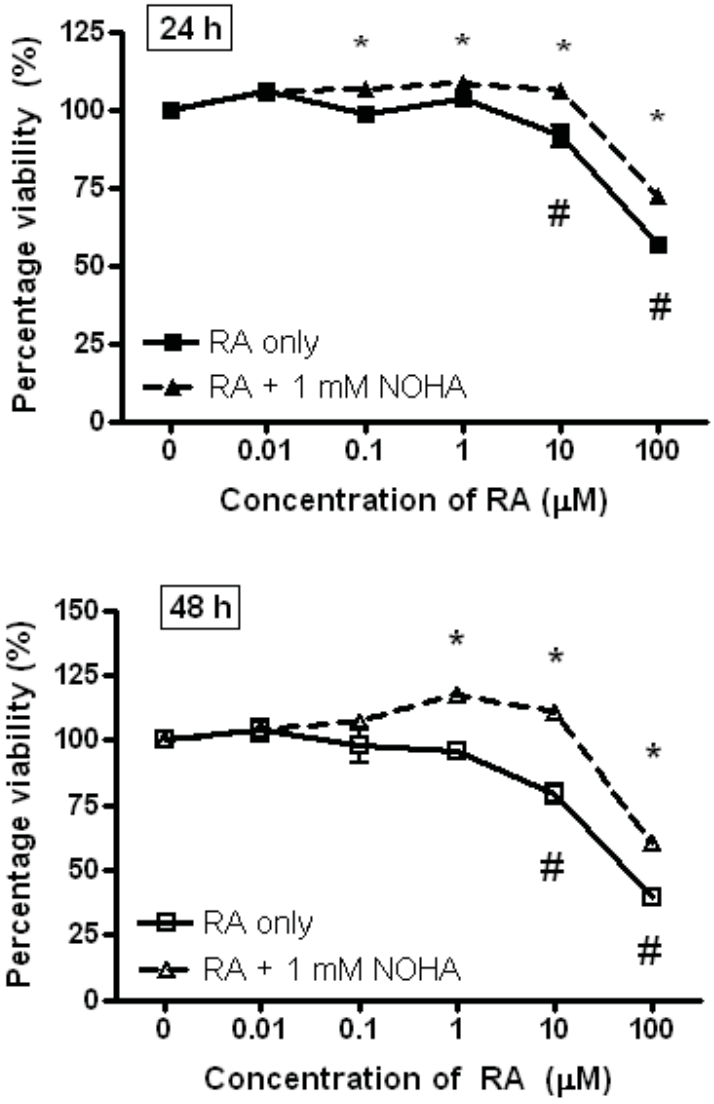
from 39 % to 60 % for 24 and 48 h, respectively (Fig 3.9B).

To determine whether arginase was the only factor involved in the RA-induced cell growth inhibition in B16 cells, norNOHA, a specific arginase inhibitor, was added to the cells in the presence of RA. In the presence of norNOHA, B16 cells recovered growth in 0.1, 1 and 10 μM RA but there were no significant differences in 100 μM RA at 24 h (Fig. 3.9C).

A


Percentage viability (%)
110
100
90
80
70
60
50
*
#
#
Control
10 μM RA
1 mM NOHA
10 μM RA + 0.01 mM NOHA
10 μM RA + 0.1 mM NOHA
10 μM RA + 0.5 mM NOHA
10 μM RA + 1 mM NOHA

B


24 h
Percentage viability (%)
125
100
75
50
25
0
RA only
RA + 1 mM NOHA
0
0.01
0.1
1
10
100
Concentration of RA (μM)
48 h
Percentage viability (%)
150
125
100
75
50
25
0
RA only
RA + 1 mM NOHA
0
0.01
0.1
1
10
100
Concentration of RA (μM)

C

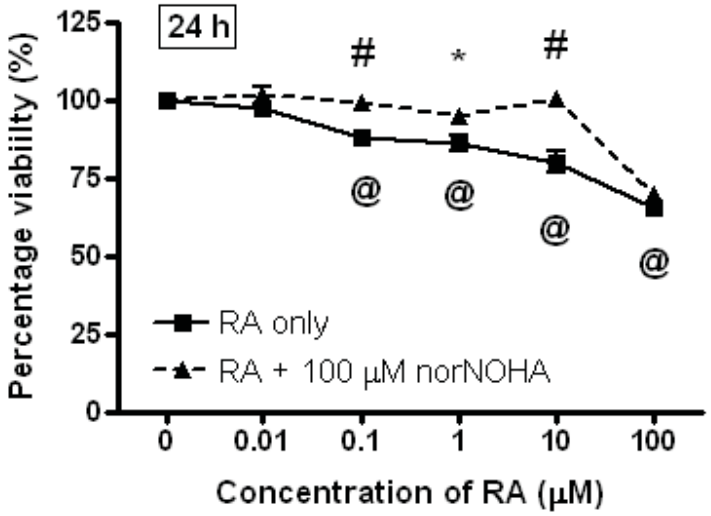


Fig. 3.9: Arginase regulates RA-mediated cell proliferation inhibition. (*A*), B16 cells were incubated for 24 h with 10 μM RA in the presence or absence of different concentrations of NOHA. Cell proliferation was detected with MTT assay. *, $p < 0.01$(vs control); #, $p < 0.05$ (vs 10 μM RA). Values are expressed as the mean ± SEM of three experiments performed in triplicate. (*B*), B16 cells were incubated for 24 h (upper) or 48 h (lower) with different concentrations of RA in the presence or absence of 1 mM NOHA. There is a significant recovery of cell proliferation when cells were treated with RA from 0.1 μM to 100 μM together with 1 mM NOHA at 24 h (#, $p < 0.01$, vs control; *, $p < 0.01$,vs RA only at the same concentration) and from 1 μM to 100 μM with 1 mM NOHA at 48 h (#, $p < 0.01$, vs control; *, $p < 0.01$,vs RA only at the same concentration) compared with treatment with RA alone. Values are expressed as the mean ± SEM of three experiments performed in triplicate. (*C*), B16 cells were incubated for 24 h with different concentrations of RA in the presence or

absence of 100 μM norNOHA. There was a significant recovery of cell proliferation when cells were treated with RA from 0.1 μM to 10 μM together with the specific arginase inhibitor at 24 h, (*, $p < 0.05$; #, $p < 0.01$ vs RA only). Values are mean ± SEM of three experiments performed in triplicate.

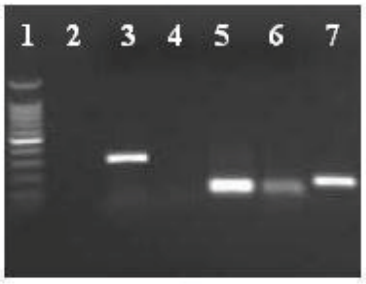
3.3 Product of arginase reaction induces cell death in P19 and B16 cells

3.3.1 Gene expression profiles of P19 and B16 cells

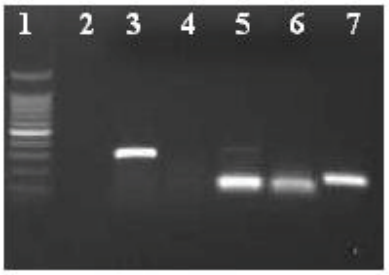
Arginase plays an important role in urea cycle by converting *L*-arginine into *L*-ornithine and urea, and *L*-ornithine acts as a common substrate of ornithine transcarbamoylase (OTC), ornithine aminotransferase (OAT) and ornithine decarboxylase (ODC). Since arginase was found up-regulated in RA-stimulated apoptosis or cell growth inhibition in P19 and B16 cells, respectively, it was interesting to examine the mechanism by which the ornithine pathway was involved. Therefore, the gene expression profiles of untreated and 10 μM RA-treated (for 24 h) P19 and B16 cells were examined by performing a RT-PCR assay and the PCR products were visualized by DNA gel electrophoresis (Fig. 3.10). The results showed that both cell lines expressed a similar gene pattern in the arginase and ornithine pathway before and after RA treatment. Both cell lines did not express a basal level of arginase I but arginase I was significantly up-regulated in P19 cells while the expression of arginase I was not observed in B16 cells by RT-PCR. Also, both cell lines did express a moderate amount of arginase II before and after RA treatment. Moreover, there was no expression of OTC in both cell lines but they both showed a strong expression of ODC and a weak expression of OAT after RA treatment. As a result, RA-induced arginase expression in P19 and B16 cells produced an increased amount of ornithine which could be the substrate of OAT and ODC

but not OTC. In addition, by looking into the reactions from OAT and ODC, ornithine is converted into glutamate and glutamic-γ-semialdehyde by OAT in a reversible reaction, while it is consumed by ODC to form putrescine and then spermidine and spermine (polyamines) by an irreversible reaction (Fig. 1.1). Therefore, the increased amount of ornithine by RA-stimulated up-regulation of arginas might mostly enter the ODC pathway because of the higher tendency by the irreversible reaction to produce putrescine, spermidine and spermine (polyamines). As a result, the abilities of polyamines to induce cell death or cell growth inhibition were examined in both P19 and B16 cells.

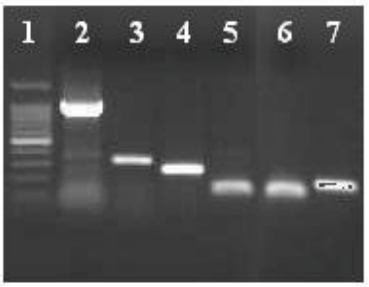
A



P19
1. 100 bp DNA ladder
2. ArgI
3. ArgII
4. OTC
5. ODC
6. OAT
7. beta-actin



B16
1. 100 bp DNA ladder
2. ArgI
3. ArgII
4. OTC
5. ODC
6. OAT
7. beta-actin



Mouse liver
1. 100 bp DNA ladder
2. ArgI
3. ArgII
4. OTC
5. ODC
6. OAT
7. beta-actin

B

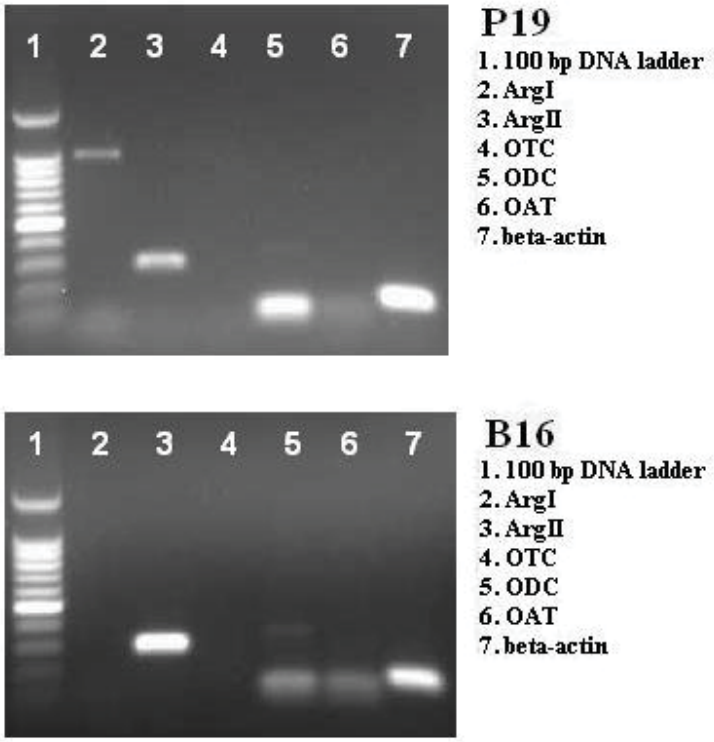


Fig. 3.10: Representative pictures of DNA gel electrophoresis of P19, B16 cells and mouse liver extract of ArgI, ArgII, OTC, ODC, OAT and beta-actin gene. 1 μg RNA was used for reverse transcription reaction and 0.4 μl first strand cDNA was used for PCR in 20 μl. (*A*), Uninduced P19 and B16 cells; (*B*), RA-induced P19 and B16 cells. Abbreviation: ArgI: arginase I; ArgII: arginase II; OTC: ornithine transcarbamoylase; ODC: ornithine decarboxylase; and OAT: ornithine aminotransferase. Beta-actin gene was used as an internal control.

3.3.2 *L*-ornithine and polyamines induce cell growth inhibition in P19 and B16 cells

Arginase was up-regulated by RA in P19 and B16 cells and therefore the concentration of ornithine was expected to increase through the conversion of arginine to ornithine by arginase. To study the effect of ornithine on P19 and B16 cells, different concentrations of *L*-ornithine was added to P19 and B16 cells and cell growth was measured after 24 h by MTT reagent. In P19 cells, a significant growth inhibition of 10.3 % was observed by adding 30 mM *L*-ornithine to the cell culture medium. At the highest concentration of *L*-ornithine tested (50 mM), there was 46 % cell growth inhibition (Fig. 3.11A). Similar results were observed in B16 cells. Significant growth inhibition (12.6 %) was detected at 25 mM of *L*-ornithine. Cell growth inhibition became severe (64.9 %) when the concentration of *L*-ornithine was 50 mM (Fig. 3.11B).

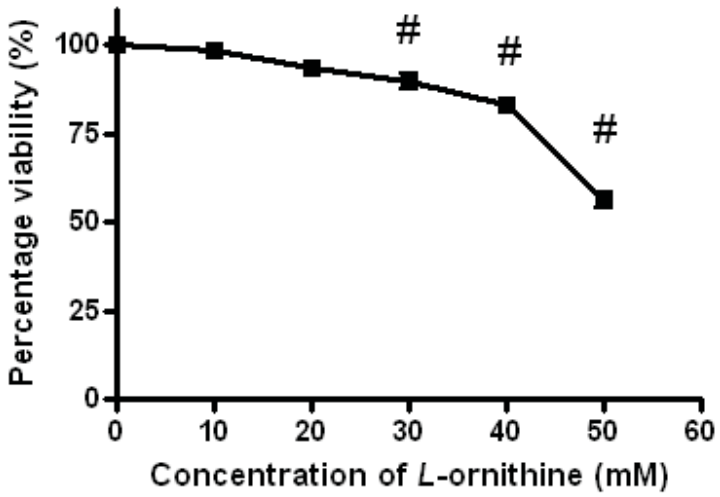
To confirm that most of the ornithine produced from the up-regulated arginase would enter the ODC pathway and cause cell growth inhibition and cell death, citrulline, product of the OTC reaction from ornithine, was added to both P19 and B16 cells. Citrulline in the same concentration as *L*-ornithine caused no growth inhibition or cell death in P19 or B16 cells (Fig. 3.12). This indicated that ornithine (produced by up-regulated arginase) did not entered into the urea cycle because neither cell lines expressed OTC, so that citrulline did not lead to cell death in either cell line. In addition, cell death might not be due to the effect of salt formation in such high concentration, but because of the

effect of *L*-ornithine itself.

As discussed above, ornithine produced by the up-regulated arginase in P19 and B16 cells is expected to enter the ODC pathway due to the irreversible reaction of ODC. Therefore, putrescine and the subsequent spermidine and spermine would be produced and accumulated in RA-treated P19 and B16 cells. To study the ability of these polyamines to induce growth inhibition in P19 and B16 cells, different concentrations of putrescine, spermidine and spermine were added to the cells for 24 h and cell viability assay was performed. Among the three tested polyamines, putrescine did not show any effect on either cell line, even when at a concentration of 100 μM (Fig. 3.12). For spermidine, there was a concentration-dependent increase in P19 and B16 cell growth inhibition. A significant cell growth inhibition (74.9 %) was detected at 12.5 μM in P19 cells. At 25 μM, spermidine induced 85 % growth inhibition, but which did not further increased at higher concentrations (Fig. 3.12A). In B16 cells, there was no significant growth inhibition induced by 12.5 μM spermidine, but there was gone 58.5 % growth inhibition when they were subjected to 25 μM spermidine. Growth inhibition became the most pronounced at 50 and 100 μM, with 93.6 and 93.7 % inhibition respectively (Fig. 3.12B). Spermine also induced a concentration-dependent cell growth inhibition in both P19 and B16 cells. P19 cells showed an enhanced growth inhibition at 12.5 μM spermine than that of the same concentration of spermidine, but like spermidine, spermine did not give further growth inhibition with a higher concentration (Fig. 3.12A). In B16 cells, 12.5 μM spermine induced 49.5 % growth inhibition and showed greater

growth inhibition with increasing concentration (Fig. 3.12B).

A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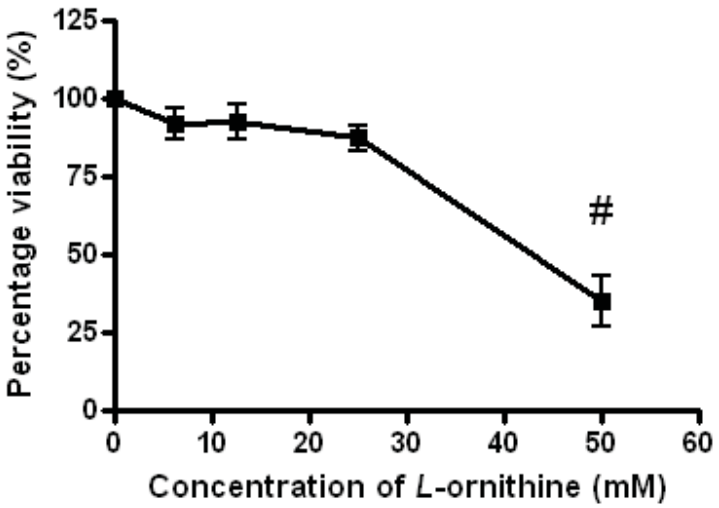
#
#
#

B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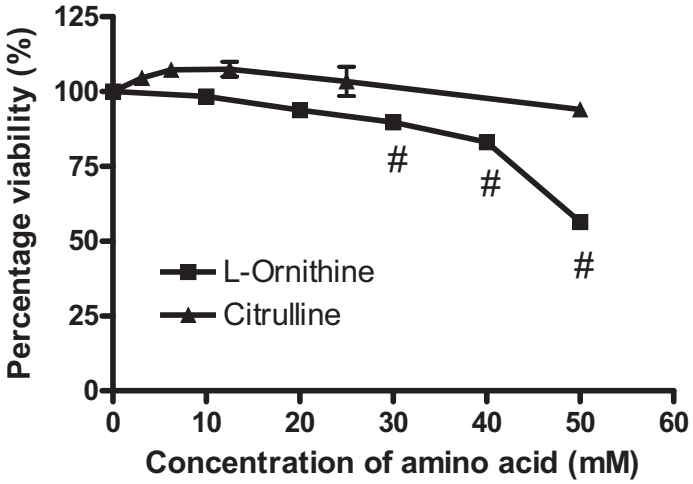

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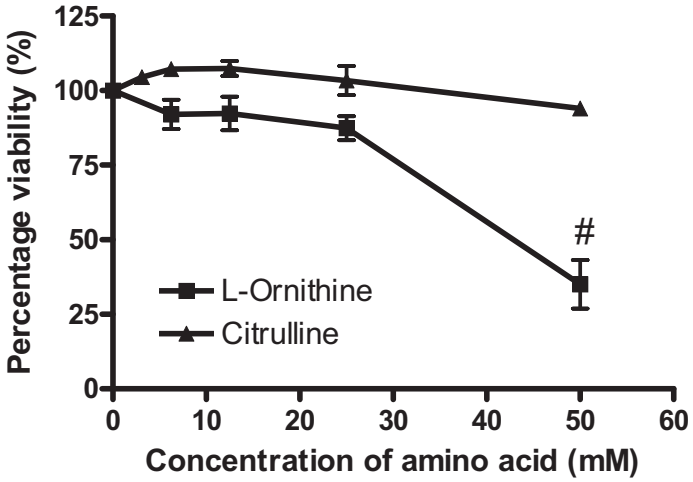
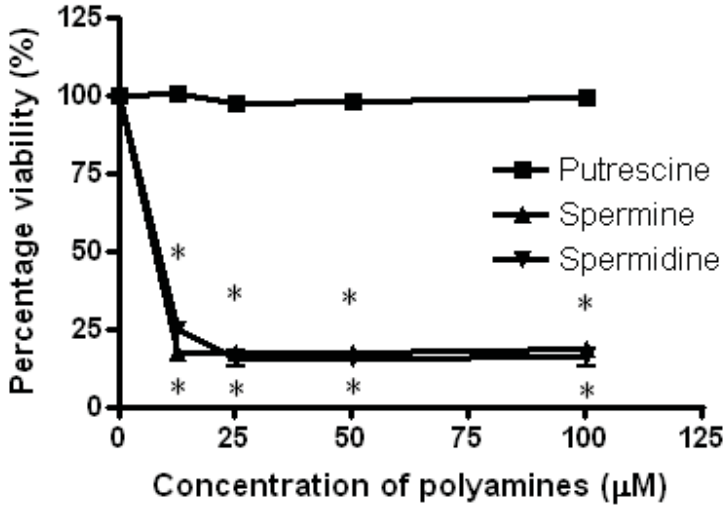


Fig. 3.11: Effect of different concentrations of *L*-ornithine or citrulline on P19 and B16 cells. P19 (*A*) and B16 (*B*) cells were treated h with different concentrations of *L*-onithine for 24. (*C*) and (*D*), effect of different concentrations of citrulline (▲) on P19 (*C*) and B16 (*D*) cells and compared with *L*-ornithine (■). Cell proliferation was then measured by MTT assay. Data are expressed as the mean ± SEM of three experiments performed in triplicate.

#, $p < 0.01$ (vs control).

A



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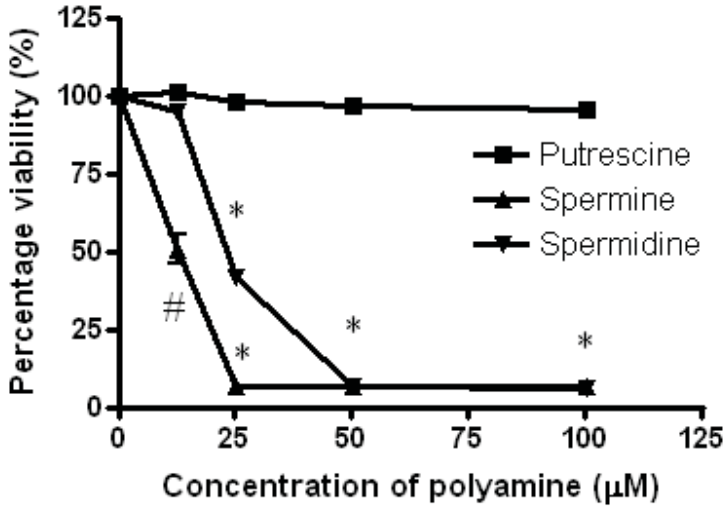


Fig. 3.12: Effect of different concentrations of various polyamines on P19 and B16 cells. P19 (*A*) and B16 (*B*) cells were treated with different concentrations of putrescine, spermidine, or spermine for 24 h. Cell proliferation was then measured by MTT assay. Data are expressed as the mean ± SEM of three experiments performed in triplicate. #, $p < 0.05$; *, $p < 0.01$ (vs control).

3.3.3 Polyamines induce apoptosis in P19 cells

For the first time, we showed that RA induced apoptosis in P19 cells by stimulating the up-regulation of the arginase I gene and the corresponding enzyme activity. We also demonstrated that the products of the arginase-ODC pathway (putrescine, spermidine and spermine) affect the cell proliferation activity of P19 cells. Therefore, the ability of polyamines to induce apoptosis in P19 cells was examined. P19 cells were subjected to different concentrations of putrescine, spermidine or spermine for 24 h and assayed for apoptosis by Annexin V-FITC staining and analyzed by flow cytometry. For spermidine, there was a concentration-dependent increase in apoptosis in P19 cells. The effect was significant even at 3.125 μM and was maximum at 50 μM (Fig. 3.13). Apoptosis was also enhanced at 3.125, 6.25 and 12.5 μM spermine but was not increased further at higher concentrations (Fig. 3.13). Putrescine did not induce any apoptosis in P19 cells, consistent with the data of MTT cell viability assay.

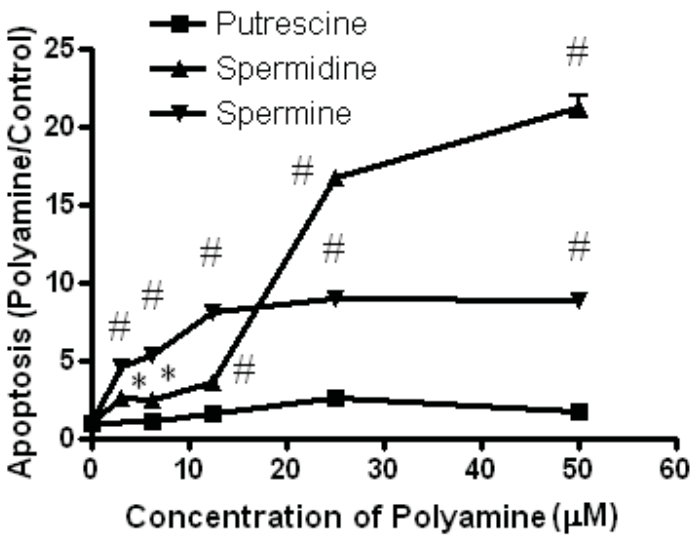


Fig. 3.13: Induction of apoptosis by polyamines in P19 cells. P19 cells were treated with different concentrations of various polyamines for 24 h. Apoptosis was then determined by Annexin V-FITC apoptosis assay (flow cytometry) using FacsAria (BD). Values are the mean ± SEM of two experiments performed in duplicate. *, $p < 0.05$; #, $p < 0.01$ (vs control).

3.3.4 Polyamines are involved in the RA-induced apoptosis in P19 cells

To determine whether RA-stimulated apoptosis in P19 cells is mediated by the formation of polyamines, effect of inhibiting the production of polyamines by DFMO, an ODC inhibitor, on RA-induced apoptosis was examined. As shown in Fig. 3.14, compared with RA-induced apoptosis in P19 cells, the apoptosis observed in the RA-treated cells was lowered by DFMO from 3.45-fold to 1.71-fold, indicating that ODC is also involved in the RA-induced apoptosis in P19 cells. Since ODC converts ornithine produced by arginase into putrescine and in turn into spermidine and spermine, the effect of polyamines on apoptosis was also studied in RA-induced apoptosis in P19 cells in the presence of DFMO. Inhibition of RA-stimulated apoptosis by DFMO in P19 cells was significantly reversed by adding spermidine or spermine (Fig. 3.14). However, putrescine did not show any reverse effect.

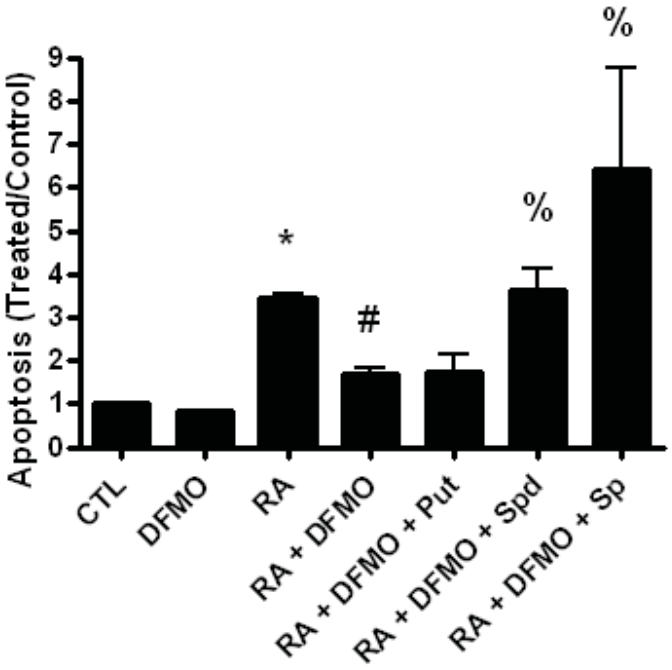


Fig. 3.14: Induction of apoptosis by polyamines in DFMO-treated P19 cells. P19 cells were treated with 10 μM RA, with or without 5 DFMO (5 mM) and 12.5 μM putrescine (put), spermidine (spd) or spermine (sp) for 24 h. Apoptosis was then determined by Annexin V-FITC apoptosis assay (flow cytometry) using FacsAria (BD). Values are the mean ± SEM of two experiments performed in duplicate. *, $p < 0.05$ (vs control); #, $p < 0.05$ (vs RA); %, $p < 0.05$ (vs RA + DFMO).

3.4 Induction of arginase and apoptosis in P19 cells by Cyclic AMP

3.4.1 Cyclic AMP induces cell death in P19 cells

It is hypothesized that arginase is important in regulating cell proliferation, cell death or apoptosis in different types of cells, and we have shown that RA-stimulated apoptosis in P19 cells or cell growth inhibition in B16 cells is arginase-dependent. To further support these new findings, cyclic AMP, another well-known arginase-inducing agent, was employed in the experiments with P19 cells.

First, dibutyrl cyclic AMP (dbcAMP) was added to P19 cells to test its ability to induce cell death in P19 cells. As shown in Fig. 3.15, P19 cells were sensitive to dbcAMP in a dose-dependent and time-dependent manner. There was no effect on cell death in the first 24 h, however, massive cell death was observed at 48 and 72 h. At 48 h, 60.85 % and 59.41 % of P19 cells died with 0.5 mM and 1 mM dbcAMP, respectively. At 72 h, 67.48 % and 59.33 % of P19 cells died with 0.5 mM and 1 mM dbcAMP, respectively.

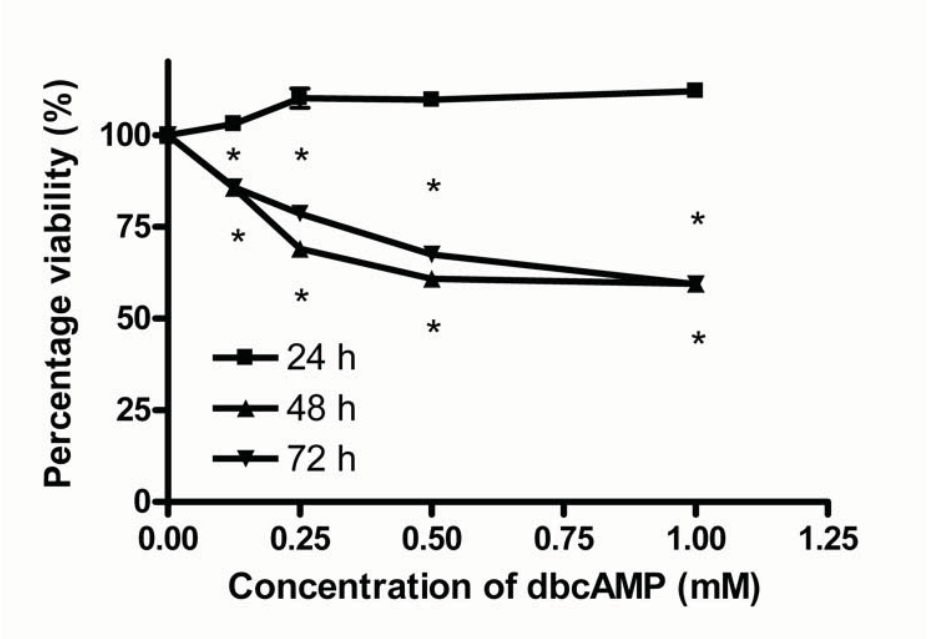


Fig. 3.15: P19 cell death induced by dibutyrl cyclic AMP (dbcAMP). P19 cells were subjected to different concentrations of dbcAMP for 24, 48 or 72 h. P19 cell death by dbcAMP was determined by MTT assay. Values are the mean ± SEM of three experiments performed in triplicate. *, $p < 0.05$ (vs control).

3.4.2 Cyclic AMP stimulates arginase activity in P19 cells

It is well known that cyclic AMP is an arginase-inducing agent in many different kinds of cell lines. To confirm that arginase was up-regulated in cyclic AMP-treated P19 cells, arginase enzyme activity was detected after the treatment for 48 h. There was no cell growth inhibition or cell death detected at 24 h post-treatment but cell death was significantly stimulated at 48 h (Fig. 3.16). After treating P19 cells with different doses of dbcAMP, there was a significant up-regulation of arginase activity by 0.5 mM and 1 mM dbcAMP at 2.15- and 2.51-fold, respectively (Fig. 3.16). At low concentrations of dbcAMP (0.0625, 0.125 and 0.25 mM), there was no change in arginase activity when compared with the control (Fig. 3.16).

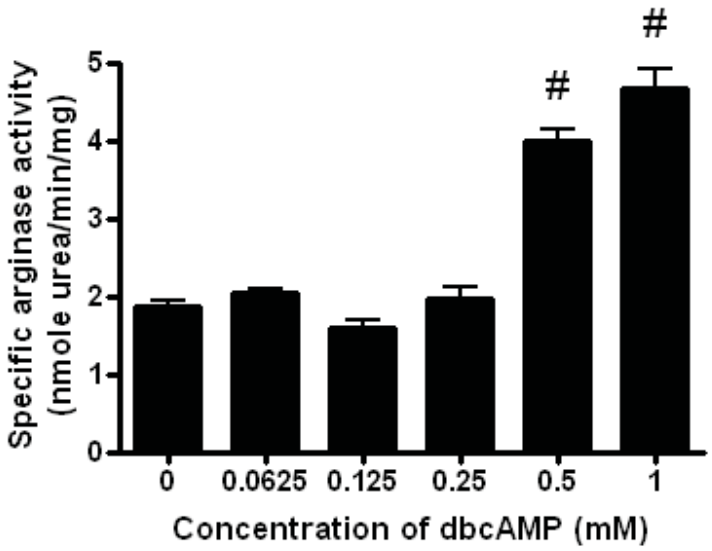


Fig. 3.16: Dose-dependent up-regulation of arginase activity in P19 cells as a result of the addition of dibutyrl cyclic AMP (dbcAMP) to culture medium. P19 cells were subjected to different concentrations of dbcAMP. At 48 h, the cells were harvested and cell lysates were assayed for arginase activity. Data represent mean ± SEM of two experiments performed in duplicate. #, $p < 0.01$ (vs control).

3.4.3 Cyclic AMP stimulates ArgI and ArgII mRNA up-regulation in P19 cells

It has long been reported that arginase activity is being induced by cyclic AMP in different kinds of cell lines. However, the isoform of arginase being stimulated is cell type-dependent. To determine which isoform of arginase was up-regulated in P19 cells by dbcAMP, *q*RT-PCR was performed after different concentrations of dbcAMP were added to P19 cells for 48 h. Both arginase I and arginase II mRNAs responded to dbcAMP in a dose-dependent manner, but arginase I mRNA showed a larger increase than arginase II (Fig. 3.17). When dbcAMP was added to P19 cells for 48 h at 0.5 mM and 1 mM, arginase I showed 13.37- and 17.79-fold up-regulation, respectively, while arginase II showed 5.28- and 6.45-fold up-regulation, respectively.

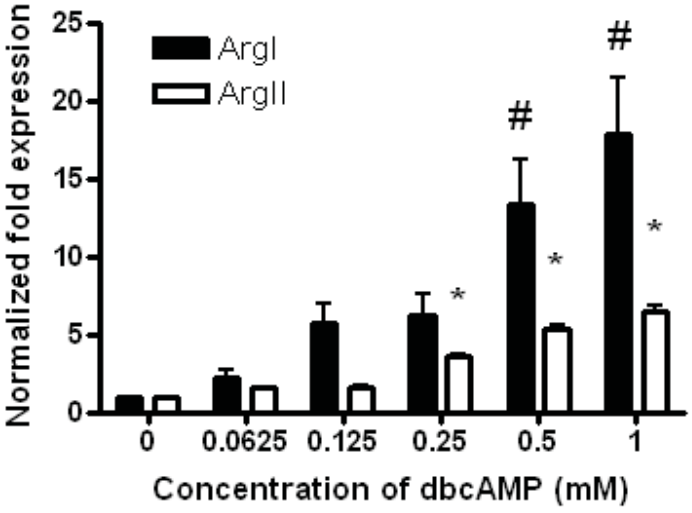


Fig. 3.17: Dose-dependent up-regulation of arginase I and arginase II mRNA expression in P19 cells as a result of the addition of dibutyrl cyclic AMP (dbcAMP). P19 cells were subjected to different concentrations of dbcAMP. At 48 h, the cells were harvested and total RNA was extracted by spin column method. 1 μg total RNA was converted into first strand cDNA and 0.4 μl first strand cDNA was used for 20 μl *q*RT-PCR. Arginase I and Arginase II mRNA expression was normalized with beta-actin mRNA expression. #, $p < 0.01$ (vs ArgI control), *, $p < 0.01$ (vs ArgII control). Data are the mean ± SEM of three experiments performed in triplicate.

3.4.4 Cyclic AMP induces arginase-dependent apoptosis in P19 cells

Since RA induced apoptosis in P19 cells was arginase-dependent, and cyclic AMP stimulated arginase activity in P19 cells, it was interesting to test if cyclic AMP could induce arginase-dependent apoptosis in P19 cells. To test the apoptosis-inducing ability of cyclic AMP in P19 cells, 0.5 mM or 1 mM dbcAMP was added to P19 cells for 48 h and apoptosis was assayed by Annexin V-PITC and PI staining apoptosis detection kit using flow cytometry. As seen in Fig. 3.18, dbcAMP at 0.5 mM and 1 mM was able to stimulate 5.34- and 11.23- fold increase of apoptosis, respectively. Then, norNOHA, a specific arginase inhibitor, was added together with 0.5 mM or 1 mM dbcAMP for 48 h and apoptosis was measured to determine whether dbcAMP-induced apoptosis in P19 cells was arginase-dependent. norNOHA itself did not show any effect on P19 cells (Fig. 3.18). When norNOHA was added together with 0.5 mM dbcAMP, apoptosis was greatly reduced from 5.34-fold to 1.58-fold when compared with the control (Fig. 3.18). However, apoptosis was not significantly reduced by adding norNOHA in combination with 1 mM dbcAMP to P19 cells. Apoptosis decreased from 11.23-fold to 9.36-fold by 1 mM dbcAMP and 1 mM dbcAMP plus norNOHA, respectively.

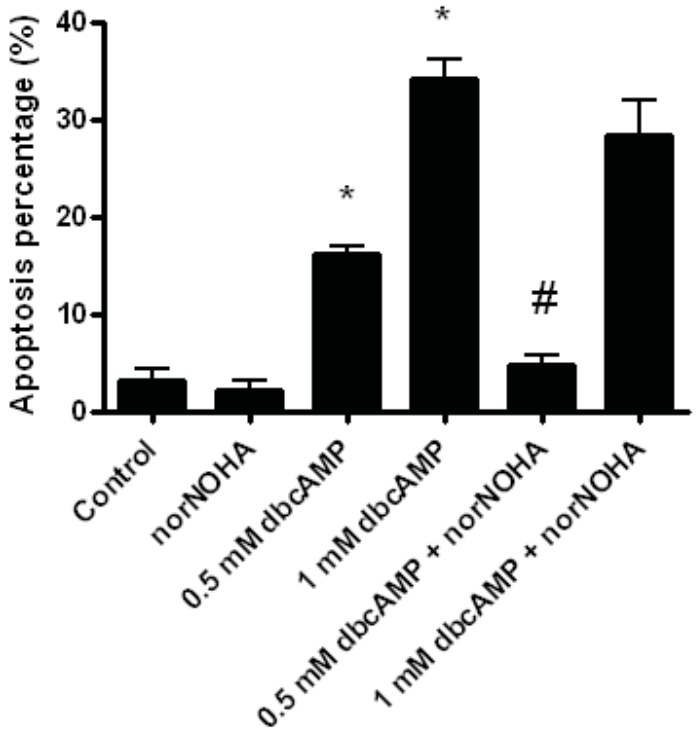


Fig. 3.18: Arginase regulates cyclic AMP-mediated apoptosis. P19 cells were incubated for 48 h with different concentrations of dibutyrl cyclic AMP (dbcAMP, 0.5 mM and 1 mM) in the presence or absence of norNOHA (100 μM). Apoptosis levels were determined by flow cytometry (Annexin V-FITC, PI staining). Values are expressed as the mean ± SEM of three experiments performed in duplicate. *, $p < 0.01$ (vs control); #, $p < 0.01$ (vs 0.5 mM dbcAMP).

3.5 Regulation of arginase I by RA in P19 cells

3.5.1 RA induces arginase I mRNA in the presence of protein synthesis inhibitor in P19 cells

RA induces arginase I up-regulation in P19 cells in a dose- and time-dependent manner. To further investigate the underlying mechanisms, experiments were performed by adding RA to P19 cells in the presence or absence of cycloheximide (CHX), a protein synthesis inhibitor, and the treated cells were subjected to *q*RT-PCR for mRNA expression analysis (Fig. 3.19). Consistent with our data, arginase I was up-regulated by 10 μM RA after 24 h. Interestingly, arginase I expression level was up-regulated by the addition of CHX alone, which suggested that some negative factors for the regulation of arginase I gene were inhibited by the protein synthesis inhibitor. Moreover, arginase I was still up-regulated by RA even in the presence of CHX, indicating that induction of arginase I gene by RA did not require *de novo* protein synthesis and was effective immediately when other factors were inhibited by the protein synthesis inhibitor. Therefore, arginase I up-regulation by RA in P19 cells was a direct effect which did not require any external factors.

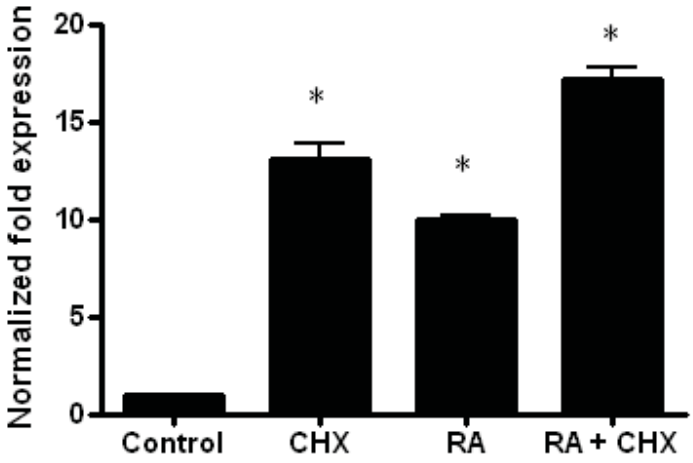


Fig.3.19: *q*RT-PCR analysis of arginase I expression in P19 cells subjected to 10 μM RA in the presence or absence of 2 μg/ml cycloheximide (CHX). P19 cells were treated with RA alone, CHX alone, or a combination of RA and CHX for 24 h. The cells were harvested and total RNA was extracted by spin column method. 1 μg total RNA was converted into first strand cDNA and 0.4 μl first strand cDNA was used for 20 μl *q*RT-PCR. Arginase I mRNA expression was normalized with beta-actin mRNA expression. The data are mean ± SEM from three independent experiments. *, p value < 0.01 (vs control).

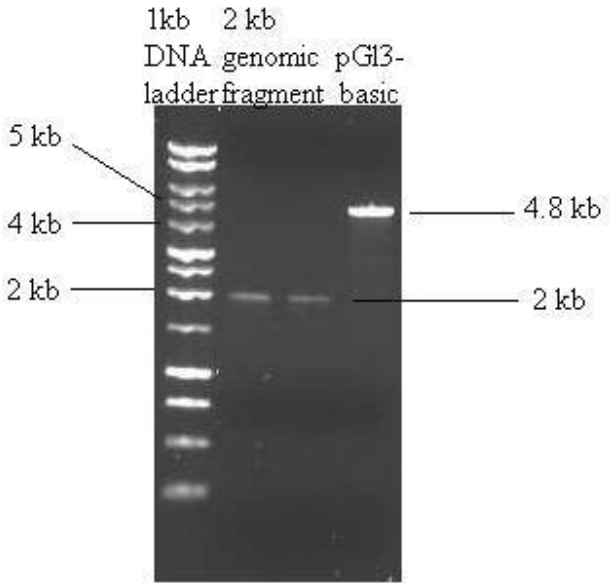
3.5.2 Construction of plasmids pGL3-AI, pGL3-AI mut RARE1 and pGL3-AI mut RARE2

After isolation of bacterial artifical chromosome (BAC) by large extract kit from the bacterial culture, 2 kb fragment upstream of the arginase I gene was amplified by PCR using a pair of specific primers, and the amplified and purified PCR product was digested by *Kpn*I and *Hind*III restriction enzymes. The restriction digested, purified genomic fragment and the vector pGL3-basic were analyzed by DNA gel electrophoresis (Fig. 3.20A). Single bands of insert and vector were found in the gel respectively, which indicated the purity of the products. The genomic fragment and the vector were then ligated by using T4 ligase and as shown by DNA gel electrophoresis, the ligation was successful because the molecular size of pGL3-AI was larger than that of pGL3-basic by about 2 kb, after both plasmids were linearized by *Kpn*I digestion (Fig. 3.20B). This indicated that the genomic DNA fragment upstream of the mouse arginase I gene was successfully inserted into the plasmid pGL3-basic to generate pGL3-AI.

By using simply text search, we found that the 2 kb genomic DNA sequence upstream of the arginase I gene contained two matches of consensus RARE. The consensus RAREs were then mutated to “switch off” the possible responsive property. To mutate the consensus sequence, a pair of specifically designed primers were used for site-directed mutagenesis with iProof high-fidelity DNA polymerase. To check the mutations, the mutated plasmids were

subjected to DNA sequencing. Since there were two consensus sequences that matched the RARE, the pGL3-AI plasmid was mutated to generate as pGL3-AI mut RARE1, pGL3-AI mut RARE2 and pGL3-AI mut RARE1 + RARE2. The sequencing results indicated the correct mutation of the two consensus RARE sequence as shown in Fig. 3.21. Although there were mismatches and gaps besides the mutated RAREs, they did not affect the experimental set up because the mismatches and gaps were not located at the possible consensus RARE sequence.

A



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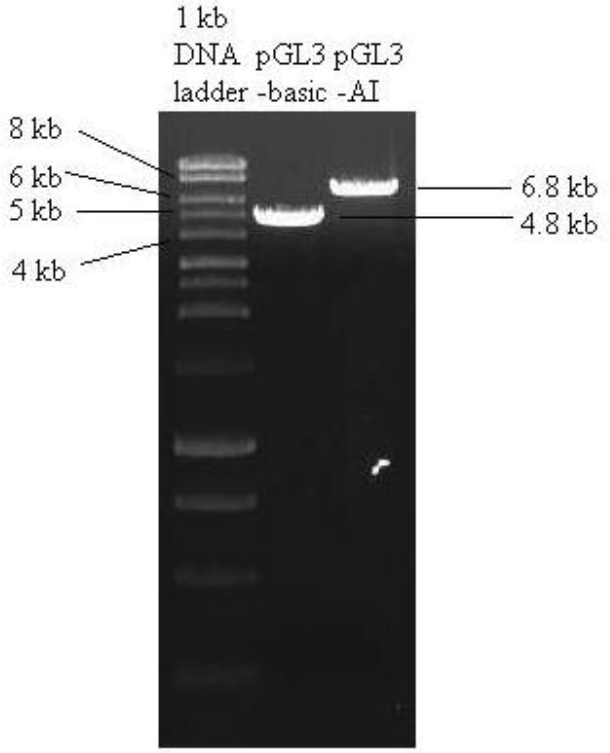

Fig. 3.20: DNA gel electrophoresis. A. Analysis of restriction enzyme digested

and purified PCR product of insert DNA, and the restriction enzyme digested and purified vector. B. Analysis of the ligation product between the genomic DNA fragment and the pGL3-basic vector. After ligation, the plasmid was transformed into *E. coli* cells. The isolated plasmids were linearized by *Kpn*I and were analyzed by DNA gel electrophoresis. The molecular size of pGL3-AI was larger than the pGL3-basic plasmid by about 2 kb.

pGL3-AI mut RARE1

-2000

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-700

pGL3-AI mut RARE1 + RARE2

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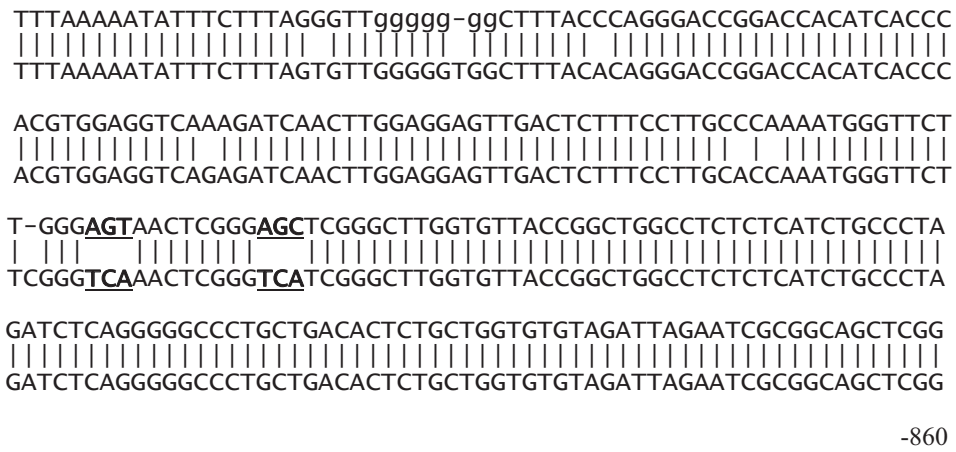


Fig.3.21: DNA sequencing results of pGL3-AI mut RARE1, pGL3-AI mut RARE2 and pGL3-AI mut RARE1 + RARE2. Wild-type pGL3-AI were subjected to site-directed mutagenesis by a specific set of primers on the putative RARE sites. After transformation and isolation of plasmid DNA, the mutated plasmids were subjected to DNA sequencing. The mutated putative RARE sites are shown in bold and underlined.

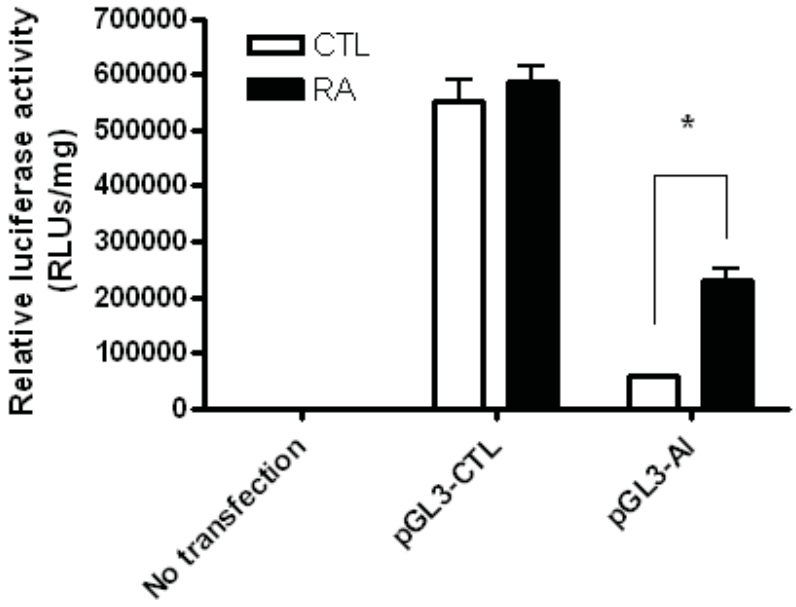
3.5.3 Induction of the arginase I promoter by RA

To investigate the transcriptional activity of the 2 kb mouse arginase I promoter sequence, we used a transient transfecton assay. The 2 kb mouse arginase I promoter sequence was amplified and subcloned into a pGL3-basic luciferase reporter vector (Fig. 2.1C). P19 cells were then transiently transfected with the wild-type promoter construct (pGL3-AI) or a positive control construct (pGL3-Control). When P19 cells were transfected with pGL3-Control, which consisted of a SV40 promoter, the firefly luciferase was constitutively expressed either in the presence or absence of 10 μM RA and a significant luciferase activity in terms of luminescence resulted. When the wild-type 2 kb genomic DNA fragment upstream mouse arginase I gene was transfected into P19 cells, addition of 10 μM RA resulted in a 4-fold marked increase in transcription activity (Fig. 3.22A). While some inducible promoter activities could be measured in the control of pGL3-AI transfected P19, the absolute level of activity was much lower than the RA group. In addition, it should be due to some leakage of the plasmid or some molecules in the culture medium were able to induce the reporter activity possibly at other response sequence.

To further examine the relationship between RA and the transcription activity of the genomic DNA sequence, different doses of RA (from 0.001 μM to 100 μM) were added to pGL3-AI-transfected P19 cells and the luciferase activity in terms of luminescence was assayed. In the presence of 0.001 μM

RA, there was no increase in luciferase activity, which indicated that at this concentration, RA was not enough to induce the promoter activity (Fig. 3.22B). However, luciferase activity was detected when RA was increased from 0.01 μM to 100 μM and addition of 10 μM RA resulted in the highest luciferase activity. These results suggested that a consensus RARE sequence was present in the 2 kb genomic DNA sequence upstream of mouse arginase I gene, and the reporter activity was dependent on the concentration of RA added.

A



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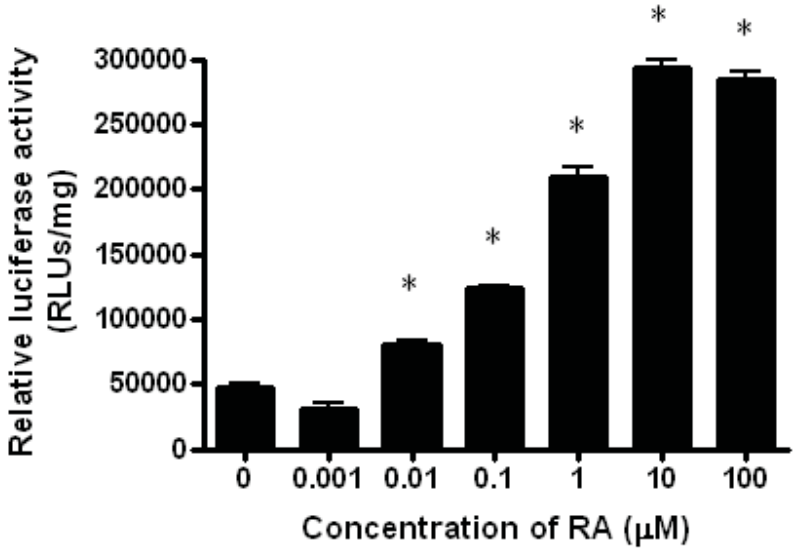


Fig. 3.22: Luciferase reporter assay on P19 cells that were transiently

transfected with the luciferase reporter plasmid. After transfection for 24 h, 10 μM RA was added into the culture and incubated for 24 h (the control was treated with vehicle solvent, which was 0.1 % absolute ethanol). Cells were then lyzed and the lysate was used for luciferase reporter assay. (*A*), P19 cells were either transfected with pGL3-CTL or pGL3-AI. Cells treated with pGL3-CTL showed a high luciferase activity in terms of luminescence in the presence or absence of RA, while cells treated with pGL3-AI showed luciferase activity only in the presence of RA but not in the absence of RA. *, p value < 0.01 (vs control of the same transfection). (*B*), P19 cells were transfected with pGL3-AI and different concentrations of RA were added to investigate the dose-dependent response of the 2 kb genomic DNA fragment. All transient transfection experiments were performed in triplicate and the data are expressed in mean ± SEM. *, p value < 0.01 (vs control).

3.5.4 Characterization of a new retinoic acid response element (RARE) in the arginase I promoter from P19 cells

To demonstrate that the putative RARE sequence(s) in the 2 kb arginase I promoter region is (are) the target for retinoic acid receptors-RA complex, we performed a luciferase reporter assay with the mutated RARE sequences using pGL3-AI mut RARE1, pGL3-AI mut RARE2 and pGL3-AI mut RARE1 + RARE2 plasmids transiently transfected in P19 cells. In the experiment, wild-type pGL3-AI, pGL3-AI mut RARE1, pGL3-AI mut RARE2 and pGL3-AI mut RARE1 + RARE2 were transiently transfected separately into P19 cells and the cell lysates were subjected to luciferase activity assays to investigate the transcription activity of each promoter. Upon the addition of 10 μM RA to the wild-type transfected P19 cells, the transcription activity was 17.62-fold higher than that without RA addition (Fig. 3.23). This indicated the possibility of a RARE sequence in the 2 kb genomic DNA sequence upstream the arginase I gene. A similar result was observed in pGL3-AI mut RARE1 wherein the addition of RA caused a 14.12-fold increase in transcription activity (Fig. 3.23), which indicated that the putative consensus RARE 1 sequence should not be a correct consensus RARE sequence. However, there was no significant increase in transcription activity in pGL3-AI mut RARE2 transfected P19 cells with the addition of RA (Fig. 3.23). This showed that with the mutation of putative RARE sequence 2, the transcription activity of the 2 kb genomic DNA sequence upstream arginase I gene was lost, indicating that the exact consensus

RARE sequence in the promoter of mouse arginase I gene was from -978 to -962 upstream of the transcription starting site of the mouse arginase I gene.

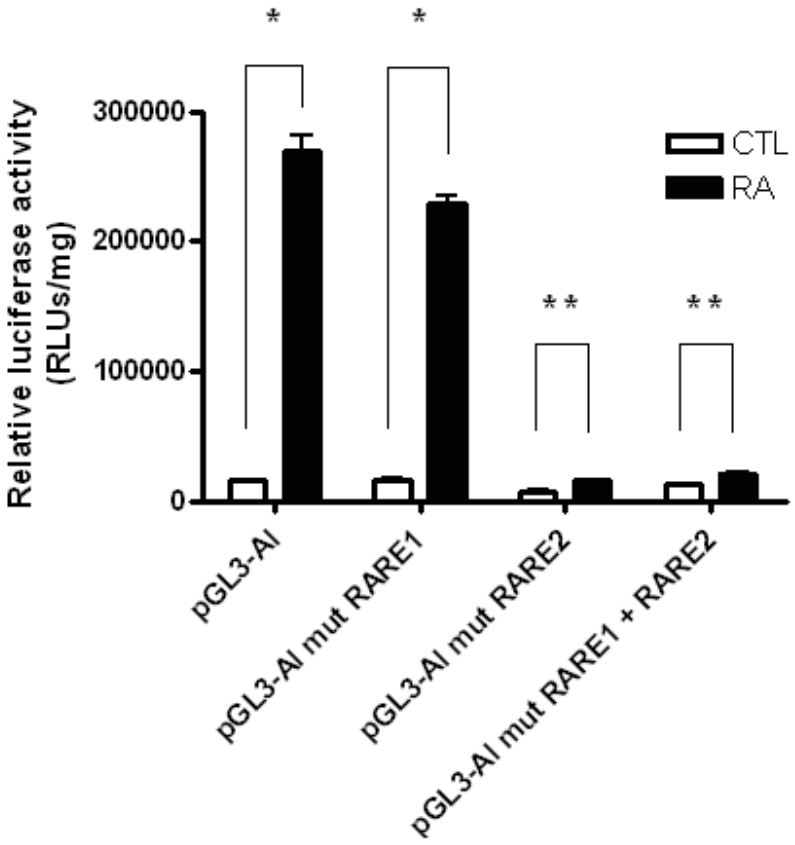


Fig. 3.23: P19 cells were transfected with pGL3-AI, pGL3-AI mut RARE1, pGL3-AI mut RARE2, or pGL3-AI mut RARE1 + RARE2 for 24 h to examine the possible presence of a RARE sequence in the 2 kb genomic DNA fragment; *, p value < 0.01 (vs the control of the same transfection); **, p value > 0.05 (vs control of the same transfection). All transient transfection experiments were performed in triplicate and the data are expressed in mean ± SEM.

3.6 Effect of bovine arginase on cells in tissue culture studies

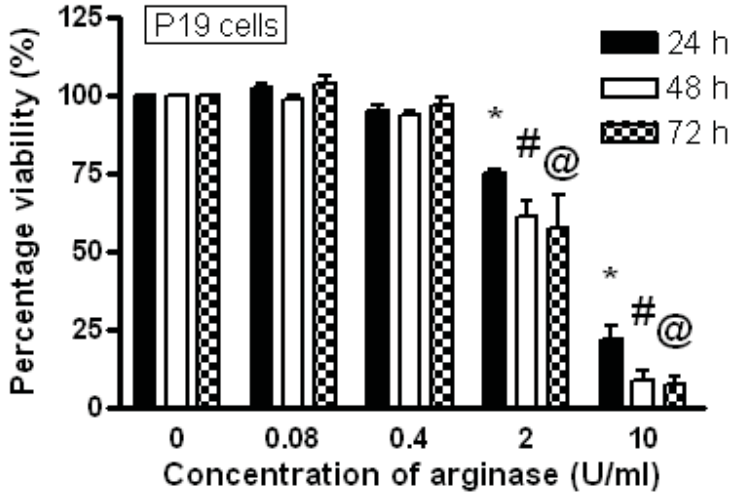
3.6.1 Bovine arginase induces cell death in P19 and B16 cells

RA induced intra-cellular arginase I up-regulation and caused arginase I-dependent apoptosis in P19 cells. Therefore, it is interesting to test whether arginase, when added extra-cellularly to the cells, can mimic the effect stimulated by RA and induce apoptosis. To address this question, bovine arginase was used in the experiment. First of all, different concentrations of bovine arginase (commercially available) were added to the culture medium of P19 cells for 24, 48 or 72 h and the effect of bovine arginase on cell growth inhibition was detected by MTT assay. At 24 h, significant cell growth inhibitions of 24.9 % and 78.2 % were observed at 2 and 10 U/ml of bovine arginase, respectively. The effect was more pronounced at 48 and 72 h. At 48 h, cell growth inhibition was 39.2 % and 91.4 % for 2 U/ml and 72 U/ml, respectively. At 72 h, cell growth inhibition was 42.9 % and 92.9 % at 2 U/ml and 72 U/ml, respectively (Fig. 3.24A).

To determine the effect of the extra-cellular addition of arginase to B16 cells, bovine arginase was added directly into the culture medium and incubated for 24, 48 or 72 h. It was found that bovine arginase could also induce cell growth inhibition in a dose-dependent manner in B16 cells. At 24 h, there was significant cell growth inhibition of 25.0 % and 71.0 % at 2 U/ml and 10 U/ml of bovine arginase, respectively. Cell growth inhibition was more obvious when

cells were incubated for a long time. At 48 h, cell growth inhibition was 73.6 % and 92.6 % at 2 U/ml and 10 U/ml, respectively. At 72 h, cell growth inhibition was 87.2 % and 93.9 % at 2 U/ml and 72 U/ml, respectively (Fig. 3.24B).

A



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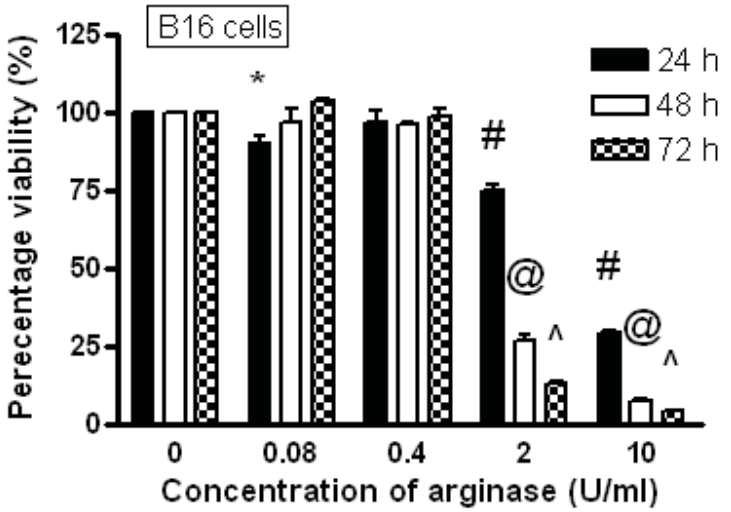


Fig. 3.24: Growth inhibiton effect of arginase (bovine arginase) on P19 (*A*) and B16 (*B*) cells. Cells were subjected to different concentrations of arginase for 24, 48 or 72 h. Cell viability was detected by MTT assay. (*A*): **, p* value < 0.01 (vs 24 h control); *#, p* value < 0.01 (vs 48 h control); *@, p* value < 0.01 (vs 72 h control). (*B*): **, p* value < 0.05 (vs 24 h control); *#, p* value < 0.01 (vs 24 h

control); @, p value < 0.01 (vs 48 h control); ^, p value < 0.01 (vs 72 h control). Data are expressed in mean ± SEM of three experiments performed in triplicate.

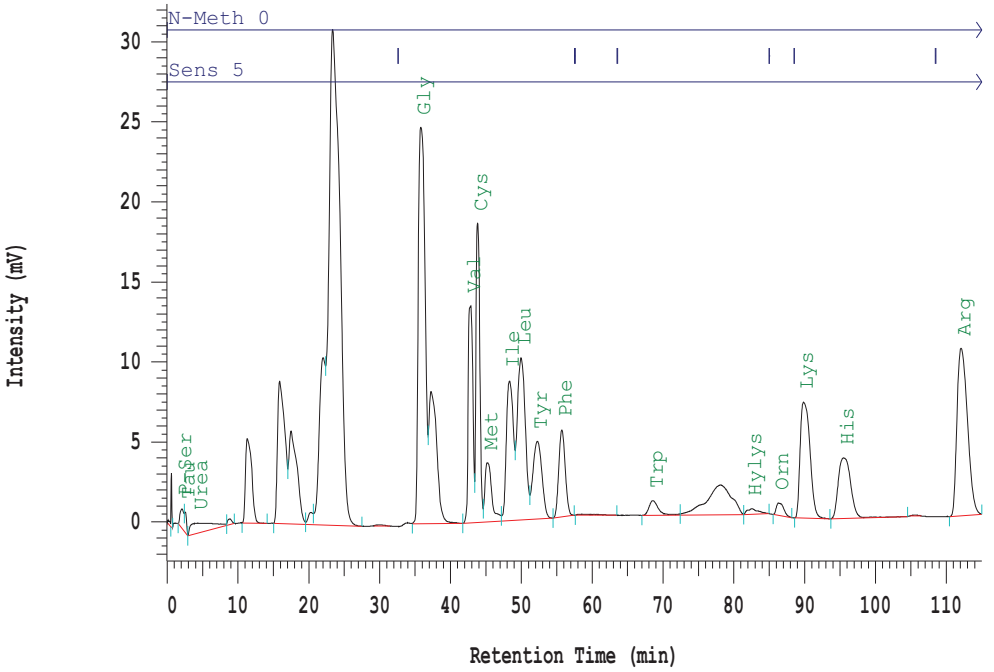
3.6.2 Bovine arginase depletes arginine and elevates ornithine in culture medium

To determine whether bovine arginase caused growth inhibition by depleting arginine and elevating ornithine in the culture medium, an amino acid analysis experiment was performed to examine the concentration of arginine and ornithine after bovine arginase was added in the culture medium. In the culture medium of P19 cells treated with 2 U/ml bovine arginase for 24 h, concentration of arginine was significantly reduced from 497.63 μM (according to manufacturers' datasheet) to 92 μM (Fig. 3.25C). There was also arginine deprivation from 497.63 μM to 355.33 μM in the control set of experiments, which might be due to the normal metabolism of the cells. Arginine was depleted to an undetectable level when 2 U/ml bovine arginase was added to P19 cells for 48 or 72 h, whereas arginine concentrations were about 290.5 μM and 300.1μM at 48 and 72 h respectively in the control experiments (Fig. 3.25C).

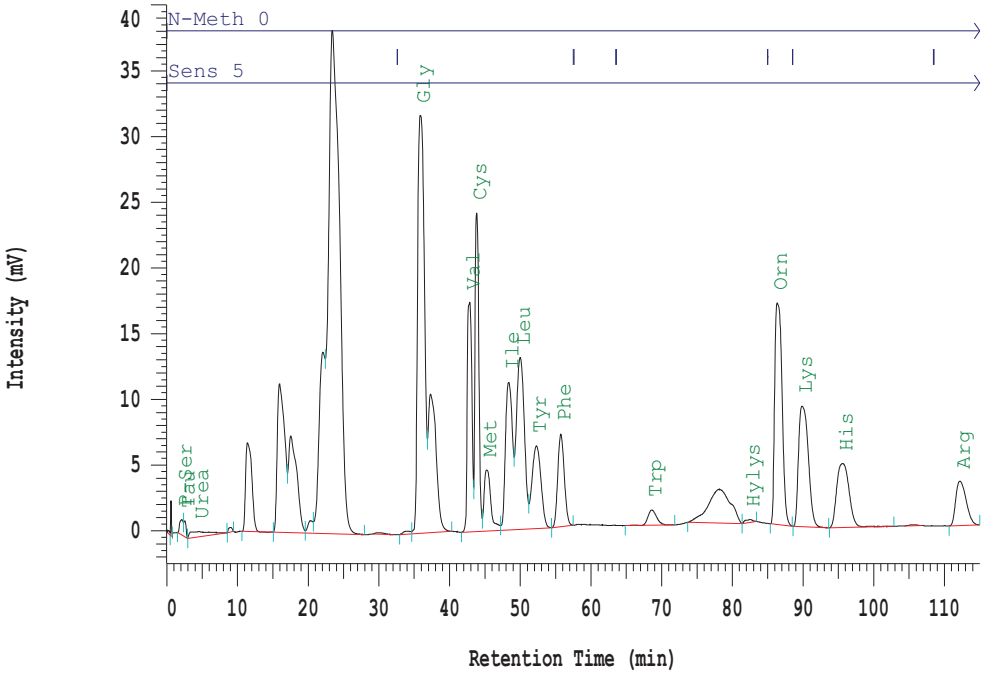
Since arginase converts arginine into ornithine and urea, there should be an increase in ornithine concentration when arginase is added to the culture medium of P19 cells. According to our amino acid analysis data, ornithine was elevated to 285.6 μM, 291.2 μM and 235.8 μM at 24, 48 and 72 h respectively when 2 U/ml bovine arginase was added (Fig. 3.25C). However, there should be no ornithine present in the non-treated culture medium according to the manufacturers' datasheet. Therefore, the elevated ornithine concentration

should be produced from the addition of arginase, which converted arginine present in the culture medium into ornithine.

A Without arginase treatment



B With 2U/ml arginase treatment



C

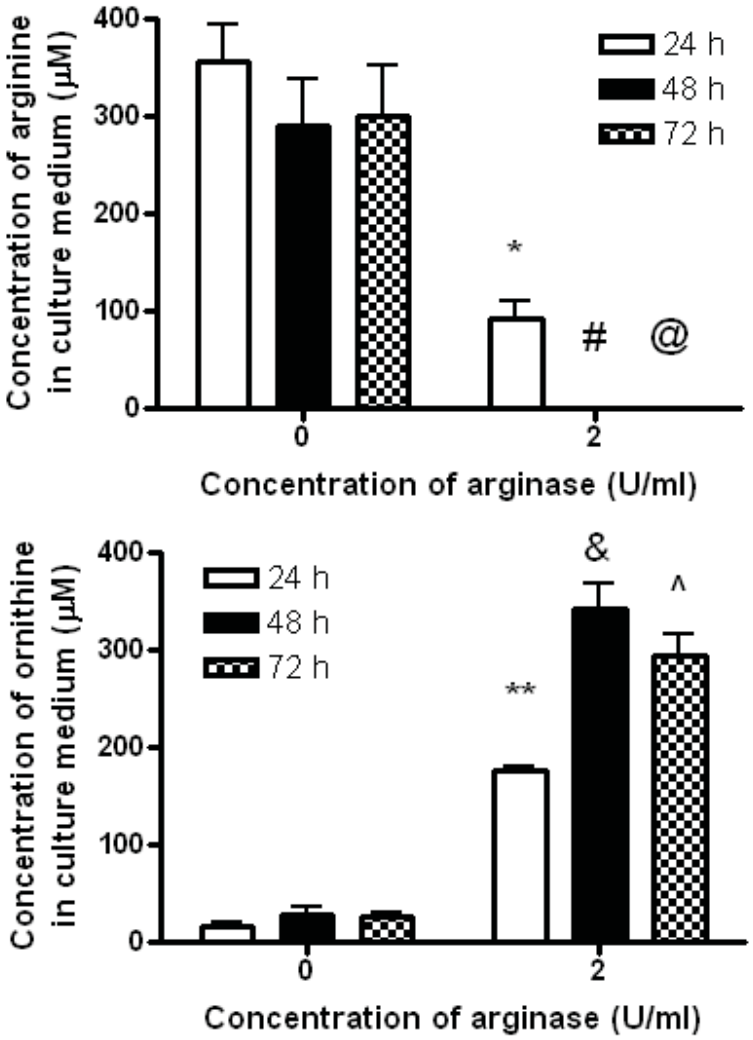


Fig. 3.25: Amino acid analysis of arginase-treated culture medium for 24 h. P19 cells were subjected to 0 U/ml or 2 U/ml arginase for 24 h. Culture media were collected and assayed for the concentration of arginine and ornithine. *A* and *B* are the representative results of amino acid analysis. (*A*), Amino acid analysis of culture medium in P19 cells without adding arginase for 24 h. (*B*), Amino acid analysis of culture medium from P19 cells with arginase added for 24 h. Abbreviation: Tau, taurine; Gly, glycine; Val, valine; Met, methionine; Ile,

isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; Trp, tryptophan; Hylys, δ-hydrpxy-lysine; Orn, ornithine; Lys, lysine; His, histidine; and Arg, arginine. (*C*), Determination of arginase concentration: *, p value < 0.01 (vs 24 h control); #, p value < 0.05 (vs 48 h control); @, p value < 0.05 (vs 72 h control); for determination of ornithine concentration, ** p value < 0.01 (vs 24 h control); &, p value < 0.01 (vs 48 h control); ^, p value < 0.01 (vs 72 h control). Values are expressed as the mean ± SEM of three experiments performed in duplicate.

3.6.3 Growth of P19 and B16 cells is arginine-dependent

Arginase converts arginine into ornithine and urea, and P19 and B16 cells undergo growth inhibition. Ornithine has been shown to be cytotoxic to both P19 and B16 cells (Fig. 3.11) but the concentration of ornithine should be at least at the millimolar level. Therefore, the effect of arginase on the two cell lines might be due to the depletion of arginine in the culture medium but not the production of ornithine. To address this question, P19 and B16 cells were subjected to different concentrations of *L*-arginine from 0 μM to 500 μM in a custom-made arginine-free culture medium for 72 h. Cell growth inhibition was determined by MTT assay. Fig 3.26 shows that both cell lines responsed to *L*-arginine in a dose-dependent manner. According to the manufacturers' formulation, normal culture medium consists of 398.1 μM and 497.63 μM *L*-arginine for the DMEM medium and alpha MEM medium respectively, and P19 cells showed 100 % or more than 100 % proliferation rate (this might be due to different formulations of medium) when the concentration of *L*-arginine was over 250 μM, whereas B16 cells showed 100 % proliferation rate when the concentration of *L*-arginine was more than 200 μM. When the concentration of *L*-arginine was lower than about 200 μM, cell growth inhibition was observed in both cell lines. Interestingly, both cell lines did not show complete growth inhibition when there was no *L*-arginine available in the culture medium. There was still a proliferation rate of 25.0 % and 26.6 % in P19 and B16 cells, respectively.

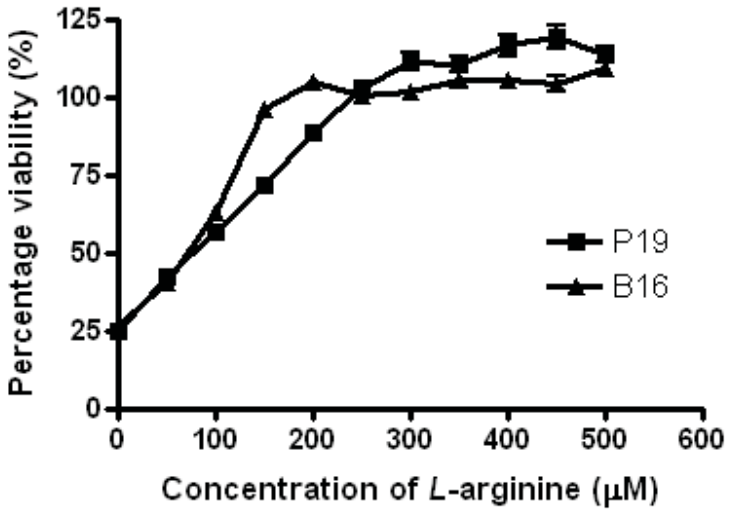
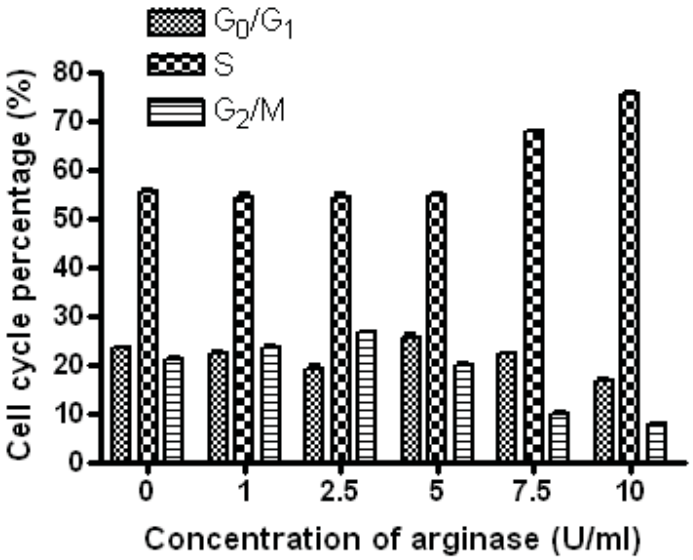


Fig. 3.26: Growth of P19 and B16 cells is arginine-dependent. Different concentrations of *L*-arginine was added to P19 or B16 cells cultured in custom-made arginine-free medium for 72 h. Cell viability was then measured by MTT assay. Values are expressed as percentage of control (cells cultured in complete medium) and are mean ± SEM of three experiments performed in duplicate.

3.6.4 Arginase stimulates cell cycle arrest in P19 and B16 cells

In order to examine the mechanism by which bovine arginase attenuates cell proliferation in P19 and B16 cells, cell cycle phase distribution was determined by staining DNA content with propidium iodine (PI) in P19 and B16 cells treated with or without bovine arginase at different concentrations and the cell cycle distribution was assayed by flow cytometry. Cell cycle phase distribution was analyzed by ModiFit LT^{TM} software. As seen in Fig. 3.27A and Table 3.1A, treatment with bovine arginase for 24 h increased the number of cells in the S phase by about 13 % and 21 % at 7.5 and 10 U/ml respectively in P19 cells, indicating that bovine arginase caused an accumulation of cells in the S phase of the cell cycle. There was not any cell cycle arrest in P19 cells with 5 U/ml bovine arginase or at a lower concentration. Although bovine arginase led to cell cycle arrest at the S phase in P19 cells, it was totally different in the case of B16 cells. Fig. 3.27B and Table 3.1B show a dose-dependent increment of cells accumulated at G_0/G_1. However, the data obtained have no significant difference in cell cycle re-distribution when compared with the control experiment.

A



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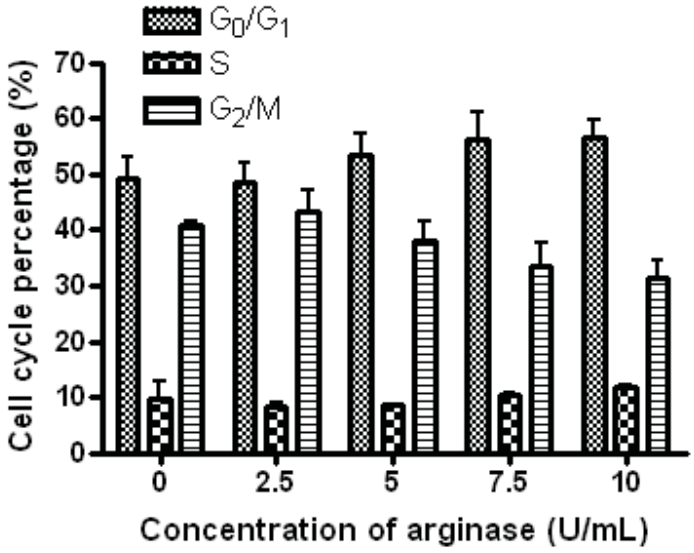


Fig. 3.27: Cell cycle analysis of arginase-treated P19 and B16 cells. P19 (*A*) or B16 (*B*) cells were treated with different concentrations of arginase for 24 h. After fixing the cells with 70 % ethanol, cell cycle distribution was examined with PI staining using FacsAria (BD). Data are mean ± SEM of two

experiments performed in triplicate.

A

Arginase concentration (U/ml)	0	1	2.5	5	7.5	10
G0/G1	23.47±0.32	22.40±0.21	19.21±0.86	25.60±0.92	22.07±0.65	16.74±0.44
S	55.44±0.64	54.54±1.23	54.31±1.28	54.61±0.72	67.95±0.09	75.74±0.83
G2/M	21.08±0.55	23.36±1.10	26.48±0.42	19.79±0.55	9.98±0.69	7.73±0.40

B

Arginase concentration (U/ml)	0	2.5	5	7.5	10
G0/G1	49.32±5.27	48.59±4.79	53.57±5.42	56.24±6.76	56.54±4.36
S	9.74±4.41	8.27±0.96	8.51±0.23	10.34±0.65	11.93±0.14
G2/M	40.95±0.86	43.14±5.76	37.92±5.18	33.42±6.11	31.53±4.21

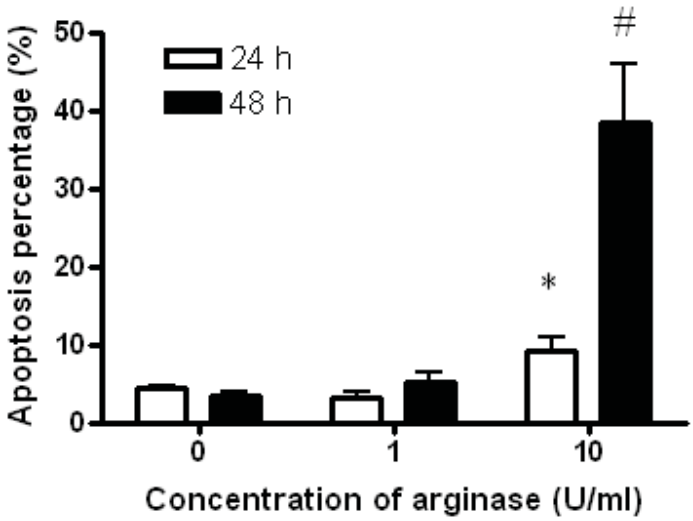
Table 3.1: Statistics of cell cycle distributions of arginase-treated P19 (A) and B16 (B) cells.

3.6.5 Arginase induces apoptotic cell death in P19 and B16 cells

To examine whether extra-cellularly added bovine arginase would mimic the effect of arginase up-regulated by RA to cause apoptosis in P19 and B16 cells, bovine arginase was added to the culture medium of P19 and B16 cells for 24 and 48 h. Fig. 3.28A shows that apoptosis slightly increased when P19 cells were treated with 10 U/ml bovine arginase for 24 h and the apoptotic effect was enhanced at 48 h in P19 cells. Low concentration of bovine arginase (1 U/ml) did not induce any significant apoptosis in P19 cells even at 48 h. The apoptosis percentages at 24 h are as follows: control: 4.46 ± 0.19 %; 1 U/ml bovine arginase: 3.26 ± 0.522 %; 10 U/ml bovine arginase: 9.26 ± 1.75 %; at 48 h, control: 3.36 ± 0.59 %; 1 U/ml bovine arginase: 5.08 ± 1.36 %; 10 U/ml bovine arginase: 38.47 ± 7.34 %.

In addition, apoptosis-inducing ability of bovine arginase was also observed in B16 cells at 48 h. Fig. 3.28B shows that there was no significant apoptosis after B16 cells were treated with arginase for 24 h even at the dosage of 10 U/ml. At 48 h, apoptotic cell death was detected at both 1 U/ml and 10 U/ml bovine arginase concentrations. The effect of 10 U/ml bovine arginase to B16 cells for 48 h was significant when compared to the control. The apoptosis percentages at 24 h are as follows, control: 1.20 ± 0.40 %; 1 U/ml bovine arginase: 1.78 ± 0.775 %; 10 U/ml bovine arginase: 4.38 ± 1.98 %; at 48 h, control: 5.50 ± 0.64 %; 1 U/ml bovine arginase: 14.20 ± 1.16 %; 10 U/ml bovine arginase: 32.78 ± 9.36 %.

A



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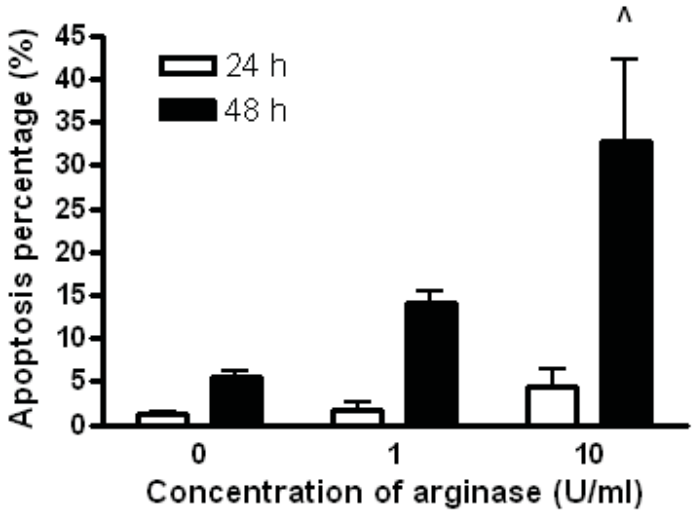


Fig. 3.28: Arginase stimulates apoptosis in both P19 (A) and B16 (B) cells. P19 and B16 cells were treated with 0 U/ml, 1 U/ml or 10 U/ml arginase for 24 or 48 h. Apoptosis was measured by Annexin V-FITC apoptosis assay (flow cytometry) using FacsAria (BD). Data are mean ± SEM of three experiments

performed in duplicate. (*A*) *, p value < 0.05 (vs 24 h control); #, p value < 0.01 (vs 48 h control); (*B*) ^, p value < 0.05 (vs 48 h control).

4 Discussion

4.1 RA induces arginase I expression and apoptosis by accumulation of polyamines in P19 cells

RA has been described to induce arginase expression in normal guinea-pig peritoneal macrophages (Rhodes and Oliver 1980). However, the mechanism of how and why arginase is up-regulated is still unknown. We hypothesized that arginase would participate in RA-induced apoptosis in P19 cells by increasing the concentration of ornithine available for the formation of polyamines by ODC. Our experiments have shown that in response to RA, expression of arginase I mRNA, but not arginase II mRNA, was up-regulated in P19 cells in a dose-dependent and time-dependent manner. The elevated arginase I mRNA expression correlated with the enhancement of arginase activity. We observed that norNOHA, a specific arginase inhibitor, which has already been shown to penetrate into cells and decrease intra-cellular arginase activity (Wei et al. 2001), and that the ODC inhibitor DFMO could abolish RA-induced apoptosis in P19 cells. This indicates that arginase and ODC activities were associated with RA-induced apoptosis in P19 cells.

Our data, similar to other publications, showed that P19 cells were sensitive to RA. Arginase I mRNA was up-regulated by RA in a concentration as low as 10 nM. This induction occurred within 12 h of RA treatment. It corresponded well to the minimum concentration and the time of exposure of

RA which efficiently induces gene modulation in P19 cells (Okazawa et al. 1996; Wu et al. 2006). In addition, our data showed that 10 μM RA gave the highest up-regulation of arginase I mRNA. This was consistent with Scheibe's findings that alkaline phosphatase activity was up-regulated maximally by 10 μM RA in P19 cells (Scheibe et al. 1991).

However, it was surprising that RA stimulated a 7-fold up-regulation of arginase I mRNA gene expression at 24 h in P19 cells (Fig. 3.1B) but there was only a 2.2-fold up-regulation in terms of arginase activity (Fig. 3.1A). Gobert (2001) made a similar observation that when macrophages were triggered by *Helicobacter pylori*, arginase II mRNA was up-regulated by 8-fold at 2 h, but arginase activity was up-regulated by 7.7-fold at 24 h (Gobert et al. 2002). One of the possible explanations to this phenomenon is that not all the mRNAs transcribed were transferred for translation or not all of them were being translated.

When arginase was up-regulated by RA, arginine would be converted into ornithine. Since arginine is a common substrate for both arginase and NOS, there is competition between arginase and NOS for the availability of arginine (Gotoh and Mori 1999). Therefore, it is of great importance to examine the expression level of NOS when arginase is up-regulated by RA. The isoform iNOS was chosen for investigation instead of endothelial NOS (eNOS) and neuronal NOS (nNOS) because eNOS and nNOS are constitutively expressed in endothelium and brain, respectively, but iNOS is induced to produce high output of NO for regulation of cell growth and apoptosis in various cell types.

Fig. 3.3 shows that there was no significant difference in the iNOS expression level in P19 cells between the control and the RA-treated cells, indicating that NO and the NOS pathway were not involved in the RA-stimulated apoptosis in P19 cells. Although iNOS was also triggered in *H. pylori*-stimulated macrophage apoptosis by the up-regulation of arginase II with the increased production of NO, the apoptosis was NO-independent (Gobert et al. 2002). Together with the data presented here, we concluded that NO is not necessarily involved in arginase-stimulated apoptosis in cells no matter what the arginase-inducer is.

It is important to note that the formation of ornithine as a substrate for ODC to produce putrescine in our studies was mainly directed from the elevated arginase metabolism pathway, because there was no ornithine present in the culture medium as shown by amino acid analysis (Fig. 3.25). When ornithine was added to the culture medium of P19 cells, a significant loss of viability was observed. Polyamines (spermidine and spermine) also induced cell growth inhibition in P19 cells. This suggested that spermidine and spermine play a role in RA-induced apoptosis in P19 cells. The experiment of treating P19 with RA in the presence of DFMO further supported this phenomenon. When ODC was inhibited by DFMO, RA-induced apoptosis in P19 cells was abolished, but spermidine and spermine restored the RA-induced apoptosis in the presence of DFMO in P19 cells. This supported the hypothesis that RA induces the up-regulation of arginase I mRNA expression and leads to the formation of ornithine, which is converted into putrescine by ODC, and the

subsequent spermidine and spermine that are responsible for the apoptotic effect in P19 cells.

When arginase was up-regulated by RA, production of ornithine should be stimulated. As discussed above, there was no ornithine present in the culture medium. Therefore, apoptosis induced by RA might be accompanied by the accumulation of ornithine. The results of the experiment of adding ornithine into P19 cells show that 30 mM ornithine stimulated a decrease in cell viability. Similar results had been reported that more than 20 mM ornithine was needed to stimulate the reduction of cell viability and cellular apoptosis in human keratinocytes (Hoche et al. 2004). In addition, since ornithine was converted into citrulline, glutamate or putrescine by OTC, OAT or ODC, the production of ornithine by arginase might then enter either one of the pathways. As P19 cells expressed only ODC and OAT even after their exposure to RA, ornithine should be converted into glutamine or putrescine only. It was believed that the majority of ornithine would enter ODC pathway because ODC was more highly expressed and the conversion of ornithine into putrescine was an irreversible chemical reaction. These factors would favor the entry of ornithine into the ODC pathway. The results of adding the ODC inhibitor DFMO in P19 cells with RA showed that ODC was dependent on RA-stimulated apoptosis, and adding polyamines (spermidine and spermine) reversed the effect of DFMO but not putrescine (Fig. 3.14). A similar phenomenon had been observed in *H. pylori*-stimulated up-regulation of arginase II and apoptosis in macrophages (Gobert et al. 2002) and over-expressed-ODC-induced apoptosis in L1210

mouse leukemia cells (Poulin et al. 1995). Oxidation stress, which results in the formation of H_2O_2 and aldehydes, the cytotoxic products of oxidative deaminations of the natural polyamines, is one of the causes of cell death in the accumulation of polyamines (Seiler and Raul 2005). Putrescine did not cause cell growth inhibition or apoptosis because catalase copes with the amount of H_2O_2 formed in the oxidation of putrescine. In addition, the aldehyde formed in the oxidation of putrescine, 4-aminobutyraldehyde, cyclises spontaneously to Δ^1-pyrroline (Seiler and Raul 2005).

However, although RA-induced apoptosis in P19 cells was arginase- and ODC-dependent, both norNOHA and DFMO could not totally abolished RA-induced apoptosis but could only reduce about half of the apoptosis induced by RA in P19 cells (Figs. 3.5 and 3.14). This indicated that polyamines (spermidine and spermine) might not be the only factor involved in RA-stimulated apoptosis in P19 cells and that other genes or molecules might also be involved. On the other hand, OAT was also expressed when RA was added to P19 cells, indicating that ornithine produced by arginase entered the OAT pathway to produce glutamine. Glutamate is converted into glutamine or γ-aminobutyric acid (GABA) (Cederbaum et al. 2004). Therefore, further experiments will be needed to examine whether glutamine or GABA stimulated apoptosis in P19 cells and whether OAT is also dependent on RA-induced apoptosis in P19 cells. In addition, the fact that other factors might also be involved in RA-triggered apoptosis in P19 cells was further supported by Wei et al. (2002), who reported that at least 5 apoptotic genes and 4 cell cycle

regulating genes are up-regulated by RA in P19 cells. This showed that apart from arginase I, there are other genes involved in the RA-induced apoptosis in P19 cells. One of the examples is the up-regulation of the mouse nuclear orphan receptor TR2-11 gene by RA (Lee and Wei 2000). It was shown that RA was able to stimulate the up-regulation of TR2-11 gene in P19 cells without any *de novo* protein synthesis, indicating that the stimulation was a direct effect. In addition, over-expression of TR2-11 protein resulted in cellular apoptosis in the absence of RA in P19 cells, showing the role of TR2-11 in regulating apoptosis in P19 cells. Hence, besides arginase I, RA is able to induce other apoptosis-regulating genes that take part in apoptosis.

4.2 RA induces arginase I and iNOS expression and cell growth inhibition in B16 cells

RA is able to induce apoptosis in P19 mouse embryonic carcinoma cells by up-regulating arginase I mRNA expression, which results in the accumulation of polyamines and causes apoptosis. It is interesting to find out whether this phenomenon can also be observed in other cell lines. B16 mouse carcinoma cells were examined because they do not have pluripotent property, which is a big difference from the P19 cells. In addition, the cancerous property of B16 cells may help us understand more about the role of RA in cancer therapy. As a result, the same strategy was employed to study the relationship between RA, arginase and apoptosis in B16 cells. Similar to P19 cells, RA stimulated arginase I up-regulation in B16 cells. However, the up-regulated arginase corresponded to cell growth inhibition but not to apoptosis in B16 cells, although polyamines were expected to be involved in the cell growth inhibition because OTC was absent in B16 cells and ODC was found more highly expressed than OAT at the DNA levels. In addition, spermidine and spermine caused a decrease in cell viability in B16 cells, indicating that polyamines were also important in regulating cell viability in B16 cells.

The data of *q*RT-PCR showed that RA induced a slight arginase I up-regulation in B16 cells at 24 h. However, DNA gel electrophoresis of RT-PCR did not show any arginase I expression even after RA was added for 24 h. This observation can be explained by the fact that the sensitivity of *q*RT-PCR is

higher than that of RT-PCR. Arginase I was up-regulated by 1.41-fold (determined by *q*RT-PCR) and the specific arginase enzyme activity was ~ 0.5 nmole urea/min/mg at 24 h of RA incubation, which was still too weak to be detected by DNA gel electrophoresis.

The data showed that the role of RA in B16 cells is different from its role in P19 cells. First, RA is able to induce apoptosis in P19 cells but it only induces cell growth inhibition in B16 cells. This observation had already been reported by Wu et al. (2005) and Estler et al. (2008) who concluded that RA is not able to induce cellular apoptosis in B16 cells(Wu et al. 2005; Estler et al. 2008). RA did not induce apoptosis in other melanoma cells like human cell line sk-mel-28 even when 10 μM RA was added to the culture medium for 8 days. It only stimulated cell growth inhibition by cell cycle re-distribution at the G_0/G_1 phase (Emionite et al. 2003).

The gene modulation patterns of different arginine-related enzymes were also different between P19 and B16 cells. At the same concentration of RA of 10 μM, arginase I mRNA was significantly induced by 7-fold in P19 cells at 24 h, but it was only 1.4-fold in B16 cells, although the data is significantly different with the control. In addition, there was a slight up-regulation of arginase II mRNA expression in B16 cells although the data is not significantly different. There was no such increase in arginase II mRNA expression in P19 cells. Moreover, it was observed that iNOS mRNA expression level was elevated when B16 cells were incubated with 10 μM RA for 24 h, indicating that iNOS might also be involved in the RA-induced cell

growth inhibition in B16 cells. In fact, when arginase inhibitor was added to B16 cells in the presence of RA, both, NOHA and norNOAH, restored the cell growth of the RA-treated B16 cells. Surprisingly, the effect of NOHA was more pronounced than that of norNOHA. At 100 μM RA, NOHA rescued 15 % cells from cell growth inhibition, while norNOHA did not show any effect. NOHA, which is an intermediate in NOS pathway and is a potent arginase inhibitor, may also inhibit the activity of NOS, while norNOHA is a specific arginase inhibitor that has no effect on NOS activity (Tenu et al. 1999). As RA also triggered iNOS mRNA expression in B16 cells, which was not observed in P19 cells (mechanism of the difference is still unknown now), iNOS might also play a role in RA-induced cell growth inhibition in B16 cells as NOHA showed a greater growth restoration than that of norNOHA in B16 cells in the presence of RA.

B16 cells also showed differences from P19 cells in terms of the sensitivity of RA on the cell viability. At 24 h, 1 nM RA was enough to stimulate a significant decrease in cell viability in P19 cells while 10 μM was needed for cell growth inhibition in B16 cells. The differences in the sensitivity of RA may be due to the fact that P19 cells have pluripotnet property for differentiation. RA is a strong differentiation agent, which has been shown in acute promyelocytic leukemia cells that it induces terminal differentiation of acute promyelocytic leukemia HL-60 cells into granulocytes, followed by cellular apoptosis. This kind of differentiation therapy using RA as a differentiating agent has been widely used in acute promyelocytic leukemia

(James et al. 1999). Therefore, a possible explanation to the differences of the sensitivity of RA between P19 and B16 cells, and to the effect of apoptosis-inducing in P19 cells and cell growth inhibition in B16 cells is that RA induces terminal differentiation and the sequential apoptosis in cells with pluripotent property rather than cells with mature phenotype. The model of RA-stimulated apoptosis in mouse embryo tail buds further supported this explanation (Shum et al. 1999). A tail bud is composed of a pool of pluripotent mesenchymal stem cells that will differentiate into almost all the tissues of the sacro-caudal body region including neural tube, notochord, paraxial mesoderm and gut endoderm in mouse embryos. Similar to P19 cells, RA induced apoptosis in tail buds, causing caudal regression in mouse embryos. Besides, it had been shown that in mouse embryonic palatal mesenchymal cells, RA induced cell cycle arrest at the G_0/G_1 phase and apoptosis in a dose-dependent manner at physiologic doses by regulating proteins involved in G_1/S phase transition (Yu et al. 2005). Therefore, RA should be more effective on cells with pluripotent property to induce terminal differentiation followed by apoptosis. In fact, many carcinoma cells have shown to have apoptosis stimulated by RA either in high concentration of RA or in long incubation time (Kim et al. 1996; Mangiarotti et al. 1997; Kawakami et al. 2006).

4.3 Induction of arginase and apoptosis by cAMP in P19 cells: confirming the role of arginase in cells

Since arginase I mRNA expression and the corresponding arginase activity were up-regulated in the presence of RA and paralleled cellular apoptosis in P19 cells, the role of arginase in P19 cells was further studied by the addition of cAMP to cells to investigate the relationship between arginase and apoptosis using cAMP as a different kind of arginase-inducer. Cyclic AMP is widely used to up-regulate arginase in many different kinds of cells including murine macrophages (Morris et al. 1998; Sosroseno et al. 2006; Haffner et al. 2008) and cultured rat hepatocytes (Edkins and Riha 1976; Nebes and Morris 1988). In all the experiments, dibutyryl cAMP (dbcAMP), a cAMP analogue, was used instead of other cAMP analogues because of its higher cell-permeablity and its non-hydrolyzable properties. In the experiments, arginase activity was up-regulated by treating P19 cells with dbcAMP for 48 h in a dose-dependent manner, and both arginase I and arginase II mRNA expressions contributed to the elevated arginase activity. Similar results had been reported by Morris (1998) who showed that cAMP was able to induce both arginase I and arginase II mRNA expression in murine macrophages, although they were using a different kind of cAMP, 8-bromo-cAMP (Morris et al. 1998). In our studies, we do not know whether arginase is directly regulated by cAMP or not, Gao (2004) had found that cAMP induced intra-cellular protein kinase A (PKA) and the subsequent activation of cAMP response element binding protein

(CREB) with the corresponding cAMP response element (CRE), stimulating the transcription of arginase in DRG neurons (Gao et al. 2004). In mouse arginase I gene, there are at least three possble consensus CRE sequences in the promoter region 2 kb upstream of transcription starting site (appendix) (Montminy et al. 1986), but experiments are needed to confirm the exact location of the CRE sequence. In addition, the Annexin V-FITC apoptotic analysis experiments show that dbcAMP stimulated a dose-dependent apoptosis response in P19 cells, and the apoptosis was arginase-dependent because simultaneously adding the specific arginase inhibitor norNOHA with 0.5 mM dbcAMP completely abolished the apoptosis (Fig. 3.18). This data supported a previous finding using RA that the up-regulation of arginase activity in P19 cells stimulated apoptosis by accumulating polyamines (spermidine and spermine). Although there were no previous data available to show that cAMP-induced arginase-dependent apoptosis in P19 cells resulted from the accumulation of polyamines, Cai (2002) showed that the up-regulation of arginase I and the subsequent synthesis of polyamines are triggered by cAMP in neuronal cells (Cai et al. 2002). Therefore, our new data suggest that the up-regulation of arginase activity in P19 cells is able to cause apoptosis by accumulating intra-cellular polyamines. It is important to note that both RA and cAMP stimulate many other genes in cells. To further support the role of arginase in apoptosis in P19 cells, over-expression of the arginase I gene in P19 cells is needed to rule out other factors, and show that over-expression of arginase I alone is enough to cause apoptosis in P19 cells.

Arginase functions by converting arginine to ornithine. Ornithine in turn can either be diverted into citrulline, glutamate or putrescine by OTC, OAT or ODC. Most frequently, ornithine is converted by ODC into putrescine and subsequent polyamines due to the wide distribution of the ODC gene in tissues. The primary importance of arginase in polyamine synthesis in macrophages (Gobert et al. 2002), neuronal cells (Cai et al. 2002) and rat aortic smooth muscle cells (Wei et al. 2001) has been described. However, the role of arginase is very different among the cells tested. In our studies, arginase was shown to be up-regulated in response to RA or cAMP and cause apoptosis in P19 cells. Similar results had been reported in murine macrophage RAW 264.7 cells. Stimulation by *H. pylori* resulted in the up-regulation of arginase II expression and apoptosis (Gobert et al. 2002). Although the modes of stimulation and the arginase types were different, both RAW 264.7 and P19 cells underwent apoptosis by the accumulation of polyamines. However, over-expression of rat arginase I mRNA in rat aortic smooth muscle cells resulted in an increment of proliferation by the accumulation of polyamines (Wei et al. 2001). In addition, over-expression of arginase was shown to overcome the inhibition by axonal growth MAG and by myelin in cerebellar and DRG neurons (Cai et al. 2002), which resulted from an elevated synthesis of polyamines. The concentrations of polyamines tested for cell viability assay or cell proliferation assay were similar among the cell lines tested. Therefore, the present data further suggest that the role of arginase and the effect of the subsequent polyamine synthesis may depend on the cell types tested in general, the effect of arginase on cell

proliferation, growth inhibition or apoptosis is cell-type specific.

4.4 Regulation of the mouse arginase I gene expression in P19 cells by RA

The role of RA in regulating genes includes activation of receptor acid receptors (RAR), binding to RAR/RAR homo-dimer to form a RA/RAR complex that recognizes a gene specific RARE to activate the transcription of the target gene (Bastien and Rochette-Egly 2004). In the process of gene regulation, no *de novo* protein synthesis is required if the target gene is directly regulated by RA. Therefore, to examine whether the mouse arginase I gene is directly regulated by RA, cycloheximide, a protein synthesis inhibitor, was added to P19 cells in the presence of RA. The arginase I mRNA expression levels were determined by *q*RT-PCR. It was found that arginase I mRNA was still expressed in the presence of cycloheximide and RA for 24 h, indicating that no *de novo* protein synthesis is required for RA-induced arginase I up-regulation and the regulation is a direct process. Interestingly, cycloheximide alone elevated the arginase I mRNA expression level, indicating that some negative factors for arginase I gene were inhibited by cycloheximide. Similar results had been obtained by Lee and Wei (2000) who showed that RA induced a direct up-regulation of the TR2-11 gene in P19 cells (Lee and Wei 2000). In addition, since the negative feedback repressors were inhibited by cycloheximide, the expression of arginase I mRNA, in the presence of RA and cycloheximide, was effectively stimulated to a higher level than that in the presence of RA alone.

To confirm the direct relationship between RA and the mouse arginase I gene expression, a luciferase reporter experiment for detecting luciferase activity in terms of luminescence has been performed. The mouse arginase promoter, which is 2 kb upstream of the transcription starting site, was chosen for analysis. First, the sequence was screened by a software which showed that there were two possible consensus RARE sequences: the first one, a sense sequence, from -978 nt to -962 nt was closer to the transcription starting site (Fig. 2.1); the other one, an anti-sense sequence, from -1853 nt to -1841 nt, was farther away from the transcription starting site (Fig. 2.1). To determine whether this 2 kb sequence contained a functional consensus RARE sequence, it was isolated and inserted into a plasmid with a luciferase reporter gene, which was then transiently transfected into P19 cells. Upon RA treatment, the luciferase signal increased when compared with those without RA treatment. This showed that the 2 kb sequence contained a consensus RARE sequence. To further confirm the exact RARE sequence in the 2 kb arginase I promoter region, the putative regions were mutated, and then the reporter assay was repeated to measure the luciferase activity. Results showed that the signal of the mutated, sense and closer consensus RARE sequence from -978 nt to -962 nt was abolished even when RA was added to the cells, but luciferase activity was still detected in the mutated sequence of the anti-sense, farther sequence from -1853 nt to -1841 nt. This proved that the sequence from -978 nt to -962 nt was a RARE sequence present in the promoter region of the mouse arginase I gene.

Although in our studies no experiment has been conducted to

investigate the expression of RAR or RXR in RA-induced arginase up-regulation in P19 cells, Song and Siu (1989) had shown that when RA was added to P19 cells, RARα and RARβ were respectively up-regulated and expressed within 12 – 24 h. RARα was kept expressed at a low level when P19 cells were not induced by RA, but was highly stimulated and the maximal level was observed after 2 h of induction with RA. On the contrary, RARβ was not expressed in uninduced condition, but was found activated in a delayed manner which expressed maximally at 12 h in P19 cells (Song and Siu 1989). Therefore, we suggest that in RA-stimulated up-regulation of arginase in P19 cells, RA might activate both RARα and RARβ, the induced RAR then interacted with RA to form a RA/RAR complex in the nucleus, which would further bind to the known consensus RARE sequence in the promoter region of the arginase I gene to activate the transcription.

However, it was surprising that direct activation of mouse arginase I mRNA expression was delayed in 12 h in RA-treated P19 cells as shown in the *q*RT-PCR experiment. In a normal gene regulation by RA in human mammary carcinoma MCF-7 cells, as early as 4 h of incubation of the cells with RA, the pro-apoptotic gene caspase-9 was up-regulated and the up-regulation did not require any *de novo* protein synthesis, indicating that the direct stimulation of the caspase-9 gene by RA was induced in a very early response (Donato and Noy 2005). The reason of the delayed up-regulation of the arginase I gene in P19 cells can be explained by the fact that cellular retinoic acid-binding protein (CRABP) the RA-transporting protein from cytoplasm to nucleus, was

expressed upon 12 h of RA treatment in P19 cells. It was observed that both the mRNA and protein levels of CRABP were up-regulated until after 12 h of RA treatment in P19 cells (Wei et al. 1989). This observation helped to explain why arginase I mRNA was expressed after 12 h incubation of RA in P19 cells even the regulation was a direct process.

4.5 Comparison of the effects between intra-cellularly induced arginase and extra-cellularly added arginase

We found that whether arginase expression was stimulated by RA or cAMP in pluripotent cells like P19, cells would undergo cell cycle re-distribution in the G_0/G_1 phase (Glozak and Rogers 2001) and apoptosis by accumulating intra-cellular polyamines (spermidine and spermine). In cells without pluripotent property like B16 cells, up-regulation of arginase would result in cell growth inhibition by cell cycle re-distribution in the G_0/G_1 phase (Niles 1987) but no apoptosis was observed. Effects of up-regulation of intra-cellular arginase greatly depend on the types of cells being treated and the mechanism is still unknown. Besides stimulating intra-cellular arginase, external arginase can be added to the culture medium as a protein drug (Esch et al. 1998; Cheng et al. 2007; Lam et al. 2009). In P19 and B16 cells, adding bovine arginase resulted in cell cycle arrest and cellular apoptosis. Unlike RA-stimulated cell cycle arrest in the G_0/G_1 phase in P19 cells, bovine arginase caused cell cycle re-distribution in the S phase, while the effects of RA and bovine arginase on cell cycle arrest was the same in the G_0/G_1 phase in B16 cells (Niles 1987). This implies that the mechanisms of intra-cellularly induced arginase and extra-cellularly added arginase on cell growth inhibition or apoptosis are different. Bovine arginase caused the depletion of arginine and elevation of ornithine (in μM level that was not enough to cause cytotoxicity as shown in Fig. 3.11), and that the growth of both P19 and B16 cells was

arginine-dependent. In addition, it had been reported that citrulline was able to rescue the recombinant human arginase-induced cell growth inhibition in hepatocellular carcinoma cells (Lam et al. 2009). These observations showed that extra-cellularly added arginase induced cell growth inhibition or cellular apoptosis by depleting arginine in the culture medium, which might be different from intra-cellular arginase-dependent cell growth inhibition or apoptosis induced by RA or cAMP.

5 Conclusions

We showed that RA induced apoptosis in the mouse P19 cells and cell growth inhibition in the mouse B16 cells by up-regulating the expression of arginase I gene. In P19 cells, RA-induced apoptosis was arginase- and ODC-dependent, indicating that the arginase-ODC pathway and the products polyamines were involved. Spermidine and spermine were found to induce apoptosis in P19 cells and decrease cell viability in B16 cells. The up-regulation of arginase I mRNA expression by RA was a direct stimulation that did not require any *de novo* protein synthesis. The luciferase activity reporter assay further showed that the promoter region of the mouse arginase I gene contained a consensus RARE sequence that was located from -978 nt to -962 nt upstream of the transcription start site. On the other hand, cyclic AMP, a well-known arginase inducer, stimulated arginase I and arginase II mRNA up-regulation and paralleled with apoptosis in P19 cells. Inhibiting arginase activity by the specific arginase inhibitor norNOHA abolished the cyclic AMP-induced apoptosis. Together with the data of RA stimulation, up-regulation of arginase in P19 cells resulted in apoptosis no matter which arginase-inducer (RA or cAMP) was used. In addition, extra-cellular arginase was added to culture medium of cells, and the effects were compared with those caused by intra-cellular arginase up-regulation. Bovine arginase induced cell cycle re-distribution and apoptosis in both P19 and B16 cells. Although the mechanisms were different when compared with those induced by RA or cyclic AMP, both

intra-cellularly induced arginase and extra-cellularly added arginase stimulated adverse effects on P19 and B16 cells, providing the insight into arginase for gene therapy or arginase protein drug in the treatment of diseases such as cancers.

6 Suggestions for future experiments

6.1 Over-expression of the arginase I gene

We showed that stimulation of the arginase I up-regulation either by RA or cyclic AMP resulted in cell growth inhibition or apoptotic cell death in the two mouse cell lines tested in our experiments. However, stimulation of gene expression by RA or cyclic AMP would not just involve arginase I only. In addition, the specific arginase inhibitor norNOHA could not totally abolish RA-induced apoptotic cell death in P19 cells, indicating that arginase was not the only factor involved in the RA-stimulated apoptosis in P19 cells. Over-expression of the arginase I gene by transfection or by other methods can be performed to investigate the sole effect of arginase up-regulation in cells. Although Wei et al. (2001) showed that over-expression of rat arginase I gene in rat aortic smooth muscle cells resulted in increased cell proliferation by accumulation of intra-cellular polyamines, the role of polyamines in regulating cell proliferation and apoptotic cell death is still controversial. Moreover, we showed in Chapter 4 that the role of arginase in cell growth and cell death is cell-type dependent. We hypothesized that cells with embryonic property (pluripotent) that are able to differentiate into other kinds of cells are more likely to undergo apoptosis in the case of over-expression of arginase, for example, RA stimulated apoptosis in P19 cells but cell growth inhibition in B16 cells. Therefore, we are going to employ mouse embryonic carcinoma P19 cells

and mouse melanoma B16 cells for the over-expression experiments to examine the different effects of arginase over-expression among different kinds of cell lines. In addition, human embryonic carcinoma cells NTera-2 will also be used for the over-expression of arginase I experiment to compare the effects of arginase over-expression between the mouse and human cell lines.

Preliminary studies showed that it is difficult to transfect or infect (by adenovirus0 P19 cells. Therefore, NTera-2 cells were used to detect apoptosis induced by over-expression of arginase I for embryonic carcinoma cells. Fig. 6.1 shows that GFP-ArgI infected NTera-2 cells showed a 18.5-fold increase in arginase activity when compared with GFP-CTL cells (from 13.2 to 244.5 nmole urea/min/mg). Next, apoptosis was determined in arginase I over-expressed NTera-2 cells by Terminal deoxynecleotidyl transferase mediated dUTP Nick End Labeling assay (TUNEL). NTera-2 cells were planted on a glass chamber slide and then subjected to infection for 24 h. TUNEL assay was then performed to detect any GFP-expressed cells showing apoptosis in GFP-ArgI construct infection (red stained cells with arginase-green fluorescent fusion protein). It was observed that upon infection with GFP-CTL construct, NTera-2 cells expressed a strong signal of GFP which was mainly localized in the nucleus of cells but they did not show any apoptotic signal of red fluorescent in the GFP-expressed cells (Fig. 6.2 C, G and K). Although there was some labeling of red fluorescent in GFP-expressed cells, the red signal was not observed in nucleus, which showed that the signal was non-specific and did not correspond to apoptosis of GFP-expressed cells (Fig. 6.2 K). On the

contrary, the GFP-ArgI infected-NTera-2 cells showed strong signals of red fluorescence in the nucleus of GFP-expressed cells (Figs. 6.2 D, H and L), showing that over-expression of arginase I results in apoptosis in NTera-2 embryonic carcinoma cells.

Previously it was shown that RA was able to induce arginase I dependent apoptosis in P19 mouse embryonic carcinoma cells but arginase I dependent cell growth inhibition only in B16 mouse melanoma cells and therefore we hypothesized that the phenomenon of arginase I-induced apoptosis could only be observed in embryonic carcinoma cells. The infection experiments showed that over-expression of arginase I in NTera-2 cells resulted in apoptosis. To support the hypothesis that arginase I over-expression does not induce apoptosis in melanoma cells, B16 cells were also infected with adenovirus bearing either GFP-CTL or GFP-ArgI construct. Results show that after infection with GFP-ArgI construct for 24 h, B16 cells did not show any apoptosis as reflected by TUNEL assay (Figs. 6.3 D, H and L) although the infection efficiency was not high when compared with the construct of GFP-CTL. The results of this experiment imply that over-expression of arginase did not result in apoptosis in melanoma cells but may only to cell growth inhibition. Therefore, MTT cell viability assay is going to be performed to determine the ability of over-expressing arginase on cell growth inhibition in B16 cells instead of any apoptotic experiment.

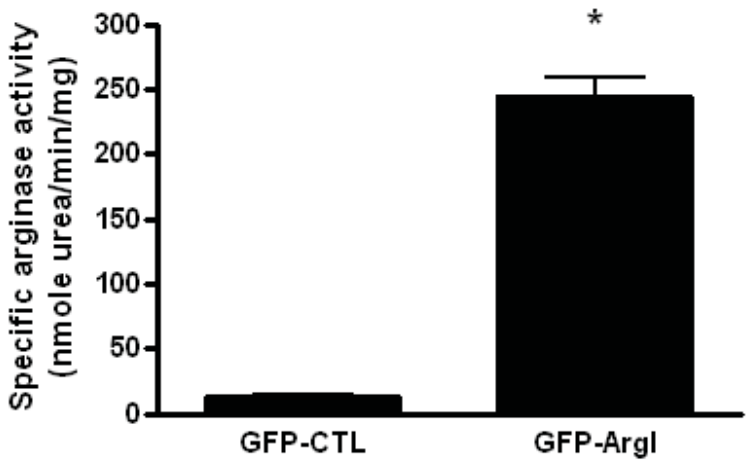


Fig. 6.1: Arginase enzyme activity of infected NTera-2 cells. NTera-2 cells were infected with either GFP-CTL or GFP-ArgI for 24 h (MOI = 100) and then harvested for arginase activity assay. Arginase enzyme activity in GFP-ArgI infected NTera-2 cells was 18.5-fold higher than that of GFP-CTL cells. *, $p < 0.05$. Data are the mean ± SEM of four independent experiments.

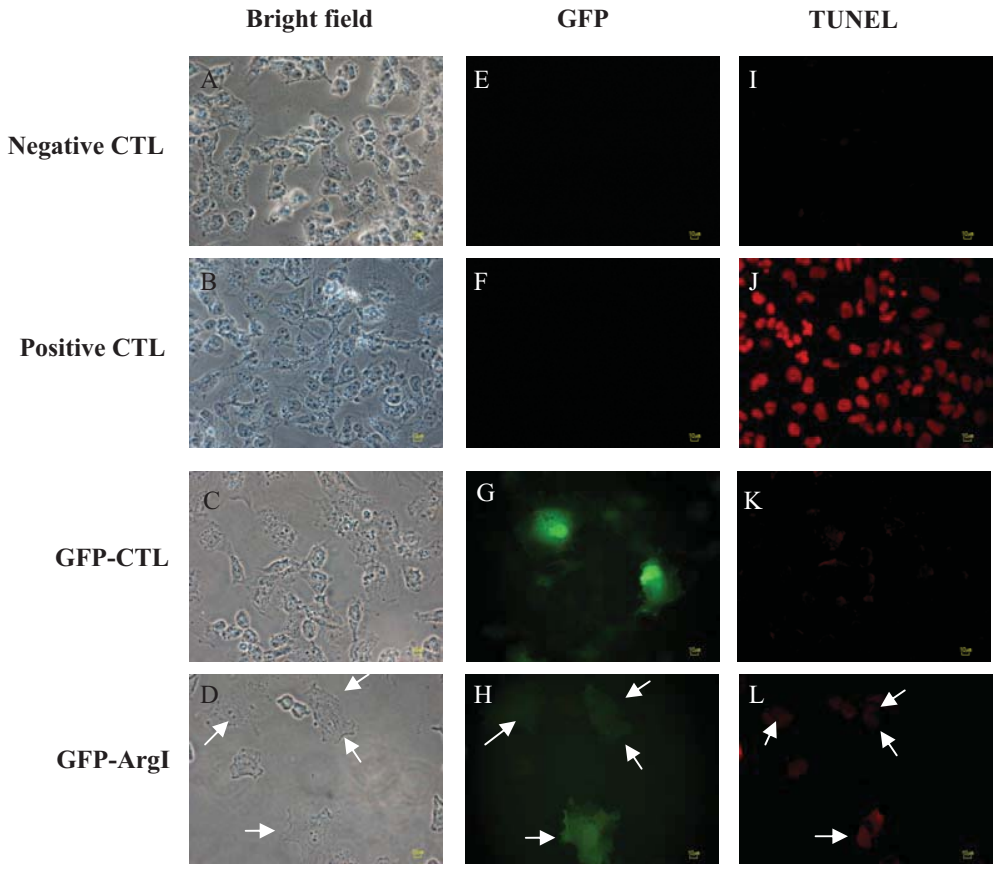


Fig. 6.2: Apoptotic assay of arginase I over-expressed NTera-2 cells by TUNEL. NTera-2 cells were infected with adenovirus bearing with either construct of GFP-CTL or GFP-ArgI for 24 h (MOI = 100) and then subjected with TUNEL assay for apoptosis. Negative control and positive control NTera-2 cells were not infected but positive control cells were treated with 3000 U/ml DNase I prior to TUNEL assay. Apoptotic cells were observed in GFP-ArgI infected NTera-2 cells (arrowheads). A, E and I: negative control cells; B, F and J: positive control cells; C, G and K: GFP-control cells; D, H and L: GFP-ArgI cells. Abbreviation: GFP, green fluorescent protein; ArgI, arginase I; arrowhead, apoptotic cells. Scale bar: 10 μm.

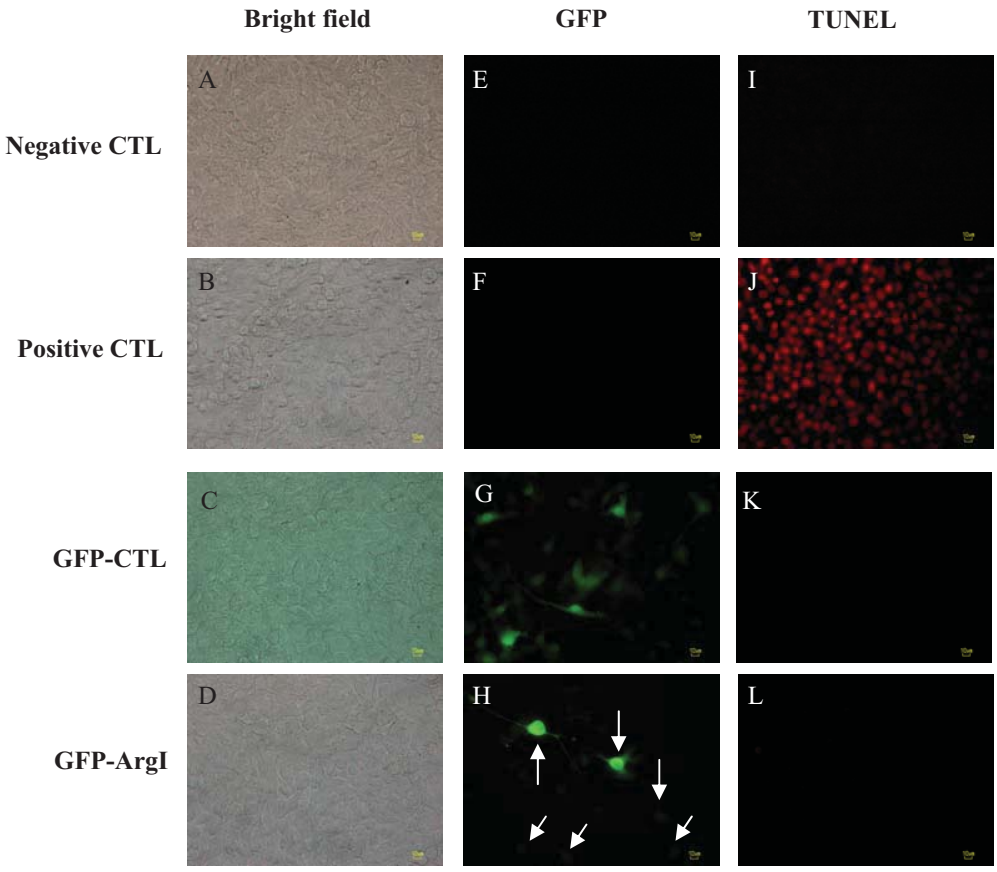


Fig. 6.3: Apoptotic assay of arginase I over-expressed B16 cells by TUNEL. B16 cells were infected with adenovirus bearing with either construct of GFP-CTL or GFP-ArgI for 24 h (MOI = 100) and then subjected with TUNEL assay for apoptosis. Negative control and positive control B16 cells were not infected but positive control cells were treated with 3000 U/ml DNase I prior to TUNEL assay. No apoptotic cells were observed in both GFP-CTL and GFP-ArgI infected B16 cells although the efficiency of infection by GFP-ArgI construct was low (H, arrowheads). A, E and I: negative control cells; B, F and J: positive control cells; C, G and K: GFP-control cells; D, H and L: GFP-ArgI cells. Abbreviation: GFP, green fluorescent protein; ArgI, arginase I; arrowhead, GFP-ArgI cells. Scale bar: 10 μm.

6.2 Possible up-regulation of arginase I gene in RA-induced human cell lines

Since the genome of mouse shares a great similarity with that of human, it will be interesting to test if similar phenomenon of RA-stimulated up-regulation of the arginase I gene would be observed in human cell lines, which then underwent cell growth inhibition or cellular apoptotic cell death. Similar to mouse B16 melanoma cells, human sk-mel-28 melanoma cells underwent cell growth inhibition when cells were subjected to RA. In addition, RA stimulated cell cycle re-distribution in sk-mel-28 cells at G_0/G_1 phase by modulation of cell cycle regulating genes (Emionite et al. 2003). This indicated that the effects of RA on either mouse or human melanoma cells are similar. Moreover, RA stimulated neuronal differentiation in human embryonic carcinoma NTera-2 cells (Lee and Andrews 1986), which shows similar pluripotent property to P19 cells. Therefore, RA-induced arginase I up-regulation in both transcriptional and post-translational levels will be determined by *q*RT-PCR and arginase activity assay, respectively, in human melanoma sk-mel-28 and human embryonic carcinoma NTera-2 cells.

6.3 Existence of consensus RARE sequence in the human arginase gene

RA induced arginase I mRNA up-regulation in mouse P19 and B16 cells, and the up-regulation of arginase I mRNA does not require any *de novo* protein synthesis, indicating that the RA-stimulated up-regulation is a direct effect. In addition, it was shown that there is a consensus RARE sequence present in the promoter region upstream of the mouse arginase I gene. Therefore, in the mouse system, RA activates the transcription of arginase I mRNA by directly binding to the corresponding RARE sequence. Since the genome of mouse shares a large similarity with that of human, and cyclic AMP activated arginase I in both human and mouse system (Morris et al. 1998; Wei et al. 2000; Sosroseno et al. 2006), and the cyclic AMP response element (CRE) is present in the human arginase I gene (Takiguchi et al. 1988). Therefore, it is interesting to investigate whether a consensus RARE sequence is present in the promoter region of the human arginase gene, provided that arginase could be up-regulated in RA-stimulated human cell lines.

6.4 RA-induced cell growth or cell death in cells by involving arginase

It has been thoroughly reported that RA is able to induce cell differentiation, cell cycle arrest and apoptosis in different kinds of cancer cells, and now it is being used as a therapeutic agent in several human cancers such as acute promyelocytic leukemia disease (Altucci and Gronemeyer 2001). In this report, we had further demonstrated that RA induced apoptosis in P19 cells through the up-regulation of arginase I expression and the accumulation of polyamines. Combined with other reports, we believed that RA was transferred by CRABPII to the nucleus (Wei et al. 1989), bound to the RARβRXR complex (Song and Siu 1989) and activated the RARE in the promoter region of the mouse arginase I gene and stimulated transcription of arginase I gene. The expression of arginase activity caused apoptosis in RA-stimulated P19 cells.

However, some reports suggested that RA stimulated proliferation in some tissues. For example, RA had a protective effect in neuronal survival (Henion and Weston 1994; Plum et al. 2001; Jacobs et al. 2006). In addition, RA was able to stimulate dermal repair and induced proliferation in basal kertinocytes (Kang et al. 1995; Zouboulis 2001). This data suggested that RA could either stimulate apoptosis in some cells or induce proliferation in other specific tissues. In 2007, Schug and his team reported that RA stimulated cell growth by the binding of alternative nuclear receptors, PPARβ/δ (Schug et al. 2007). They showed that RA stimulated cell growth with the aid of transport with FABP5 and by the binding of RA with PPARβ/δ in keratinocytes.

Therefore, they suggested that RA induced either cell proliferation or apoptosis by mode of expression of the binding proteins and the nuclear receptors in the specific cells.

In addition to Schug's report, Gallardo-Soler and his team showed that arginase I could be induced by modified lipoproteins by the activation of PPARγ/δ in macrophages (Gallardo-Soler et al. 2008). They also suggested that there was a proxisome proliferator response element in the promoter region of mouse arginase I gene. In addition, Wei and his team found that over-expression of arginase I gene stimulated the accumulation of polyamines and resulted cell proliferation in rat aortic smooth muscle cells (Wei et al. 2001). Together with the data of Schug and Gallardo-Soler, we hypothesize that RA may be able to stimulate cell proliferation in some cells by the activation of PPAR, and since these is a peroxisome proliferator response element in the promoter region of arginase I gene, the activation of PPAR by RA may cause the expression of arginase I and result cell proliferation in some cells. Although arginase I may be involved in the two different pathways with different outcomes, the effect of arginase may account to the accumulation of polyamines. It had been reported that polyamines could either cause cell growth or apoptosis in different cells and the fate was depended on the concentration of polyamines and was specific to cell types (Thomas and Thomas 2001). As a result, it is possible that RA stimulates arginase I expression and the resulting polyamines accumulation may cause either cell growth or apoptosis. Therefore, in order to prove this hypothesis, a rat aortic smooth muscle cell, A7r5, should

be employed because it has been reported that over-expression of arginase I gene increases cell proliferation in this cells. Similar to the experimental approach used for P19 and B16 cells, *q*RT-PCR for arginase I gene, arginase activity assay, cell viability assay are going to be performed. To check whether arginase induced by RA is dependent on RA-stimulated cell proliferation in A7r5 cells, the specific arginase inhibitor norNOHA will also be added together with RA.

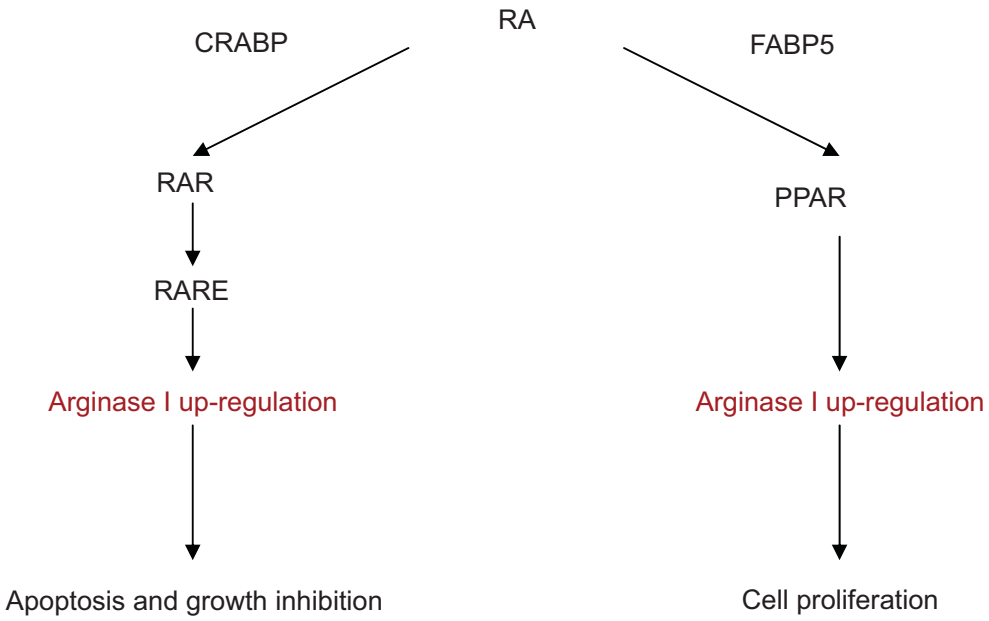
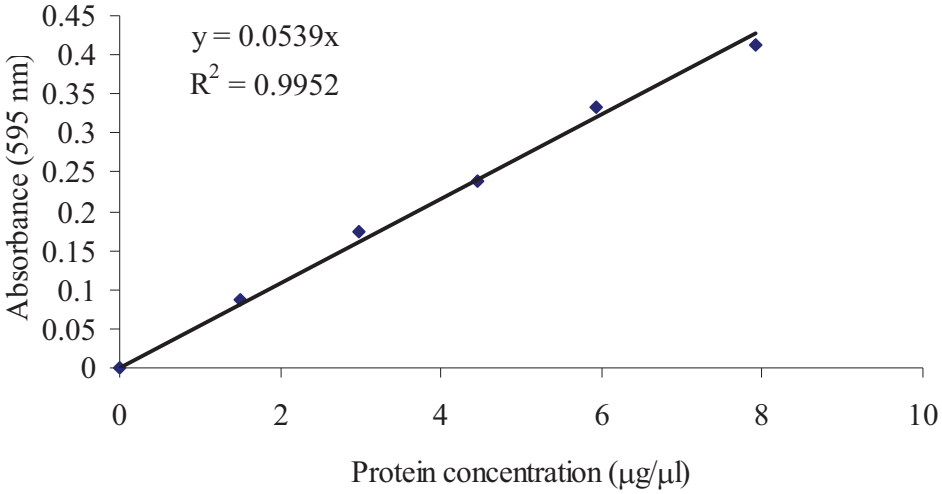


Fig. 6.4: Overview of RA, arginase, cell proliferation and apoptosis in cells.

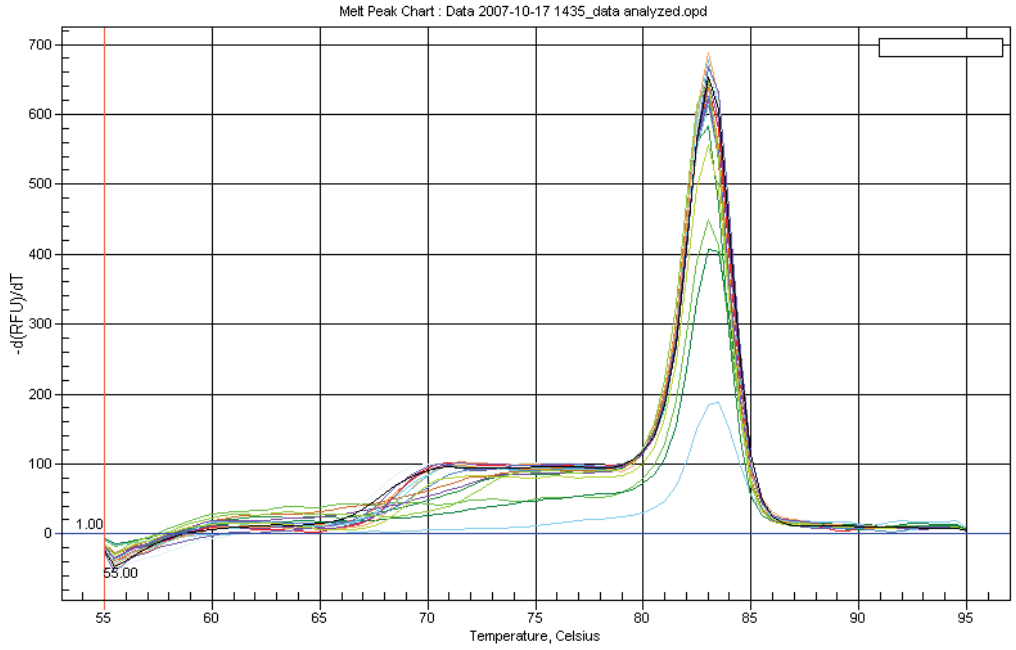
Appendix

Protein standard curve (Bradford assay)

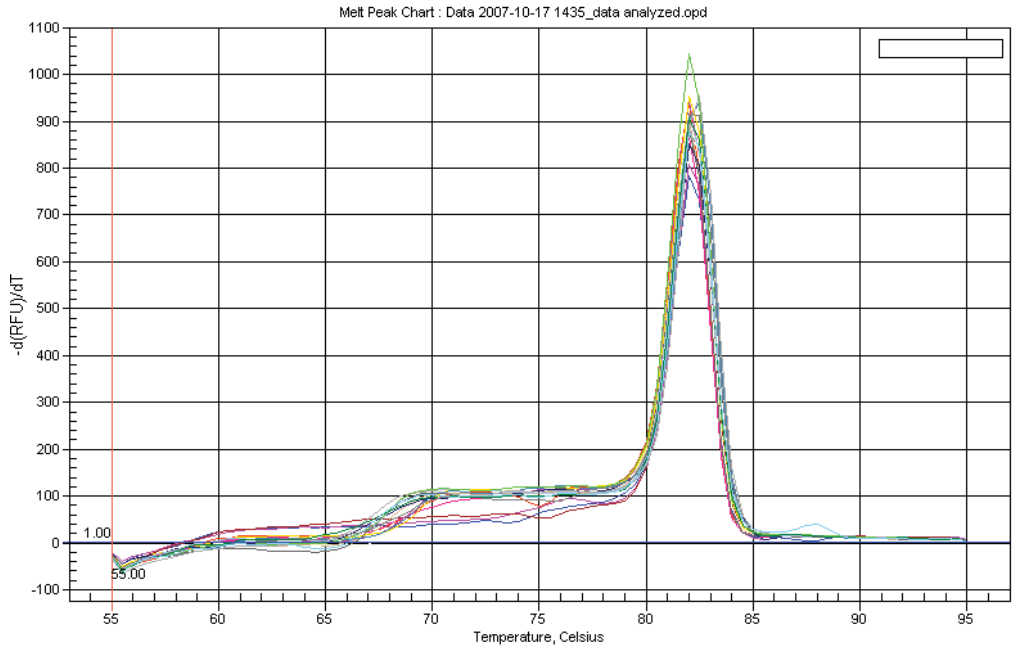


App Fig. 1: Representative result of protein standard curve by using bovine serum albumin (BSA) as the standard.

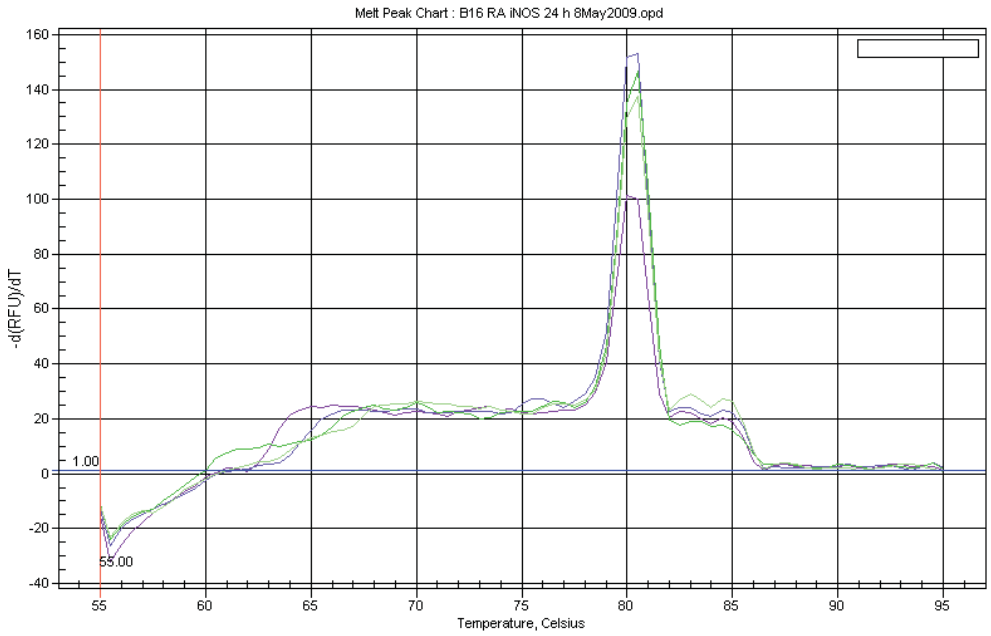
A


Melt Peak Chart : Data 2007-10-17 1435_data analyzed.opd
-d(RFU)/dT
Temperature, Celsius
1.00
55.00

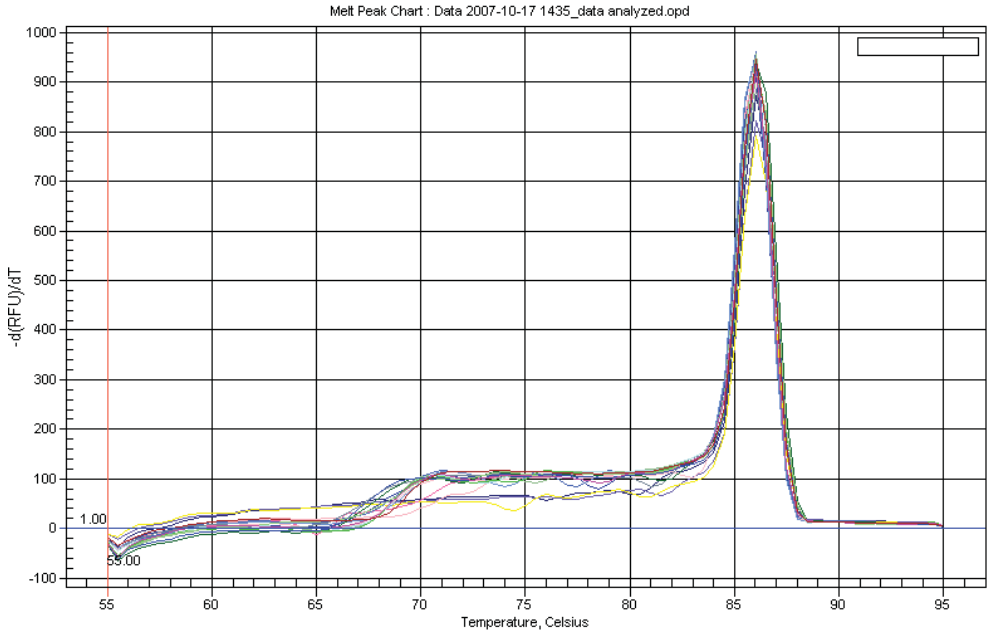
B


Melt Peak Chart : Data 2007-10-17 1435_data analyzed.opd
-d(RFU)/dT
Temperature, Celsius
1.00
55.00

C



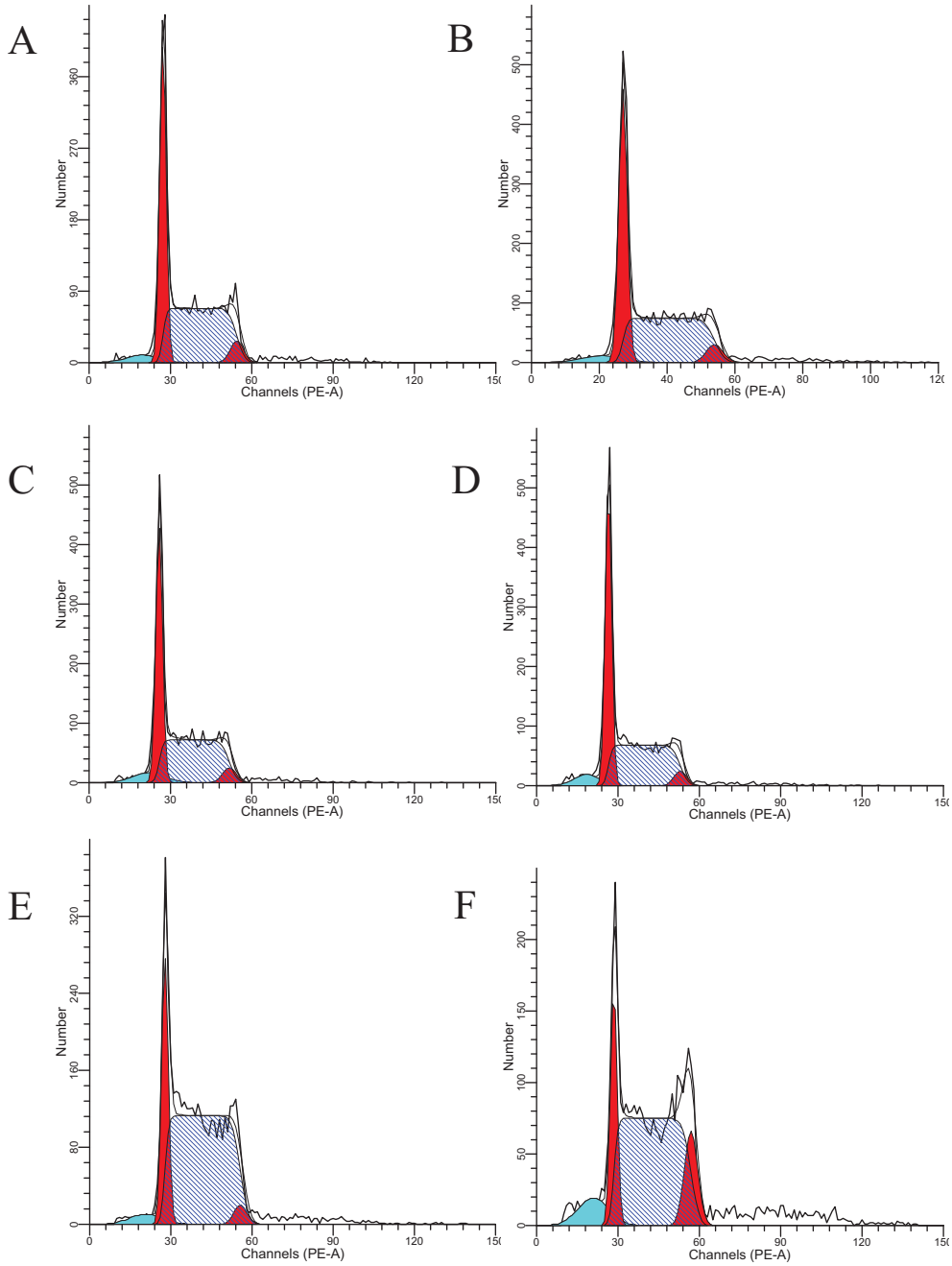
D



App Fig. 2: Melting curve analysis was performed to test the specificity of the primers used in *q*RT-PCR assay of mouse cell lines. A: Arginase I; B: Arginase II; C: iNOS; D: beta-actin.

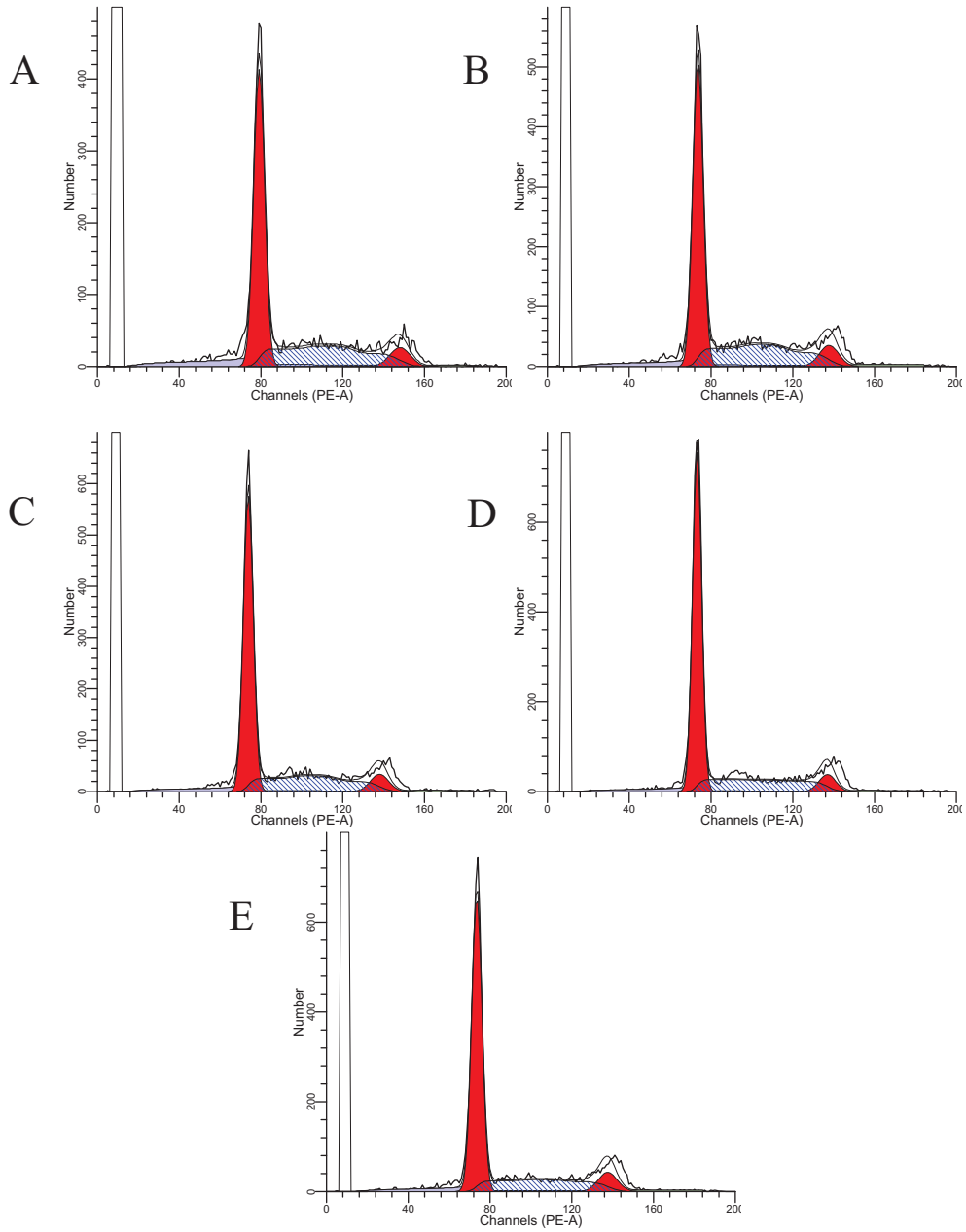
CRE consensus:	TGACGTCA
Mouse arginase I gene:	-1637 TGAGTTCA -1630
	-744 TGAAGTTA - 737
	-250 TGAGGTTA – 243

App Fig. 3: Similarities between promoter sequences of the mouse arginase I gene and the consensus sequence for the cAMP response elements (CRE consensus). Matches nucleotides are emphasized by °.



App Fig. 4: Representative histograms of the cell cycles of bovine arginase-treated P19 cells for 24 h. P19 cells were stimulated with dose-dependent bovine arginase for 24 h. Cells were harvested, fixed in 70 % ethanol overnight and then stained with PI. Cell cycle distributions were assessed by FACSAria flow cytometry and data were analyzed by using Modfit LT^{TM} software. A,

Control; B, 1 U/ml; C, 2.5 U/ml; D, 5 U/ml; E, 7.5 U/ml; and F, 10 U/ml. Blue area: apoptosis; red area: G_0/G_1 and G_2/M phase; blue shaded area: S phase.



App Fig. 5: Representative histograms of the cell cycles of bovine arginase-treated B16 cells for 24 h. B16 cells were stimulated with dose-dependent bovine arginase for 24 h. Cells were harvested, fixed in 70 % ethanol overnight and then stained with PI. Cell cycle distributions were assessed by FACSAria flow cytometry and data were analyzed by using Modfit LT^{TM} software. A,

Control; B, 2.5 U/ml; C, 5 U/ml; D, 7.5 U/ml; and E, 10 U/ml. Red area: G_0/G_1 and G_2/M phase; blue shaded area: S phase.

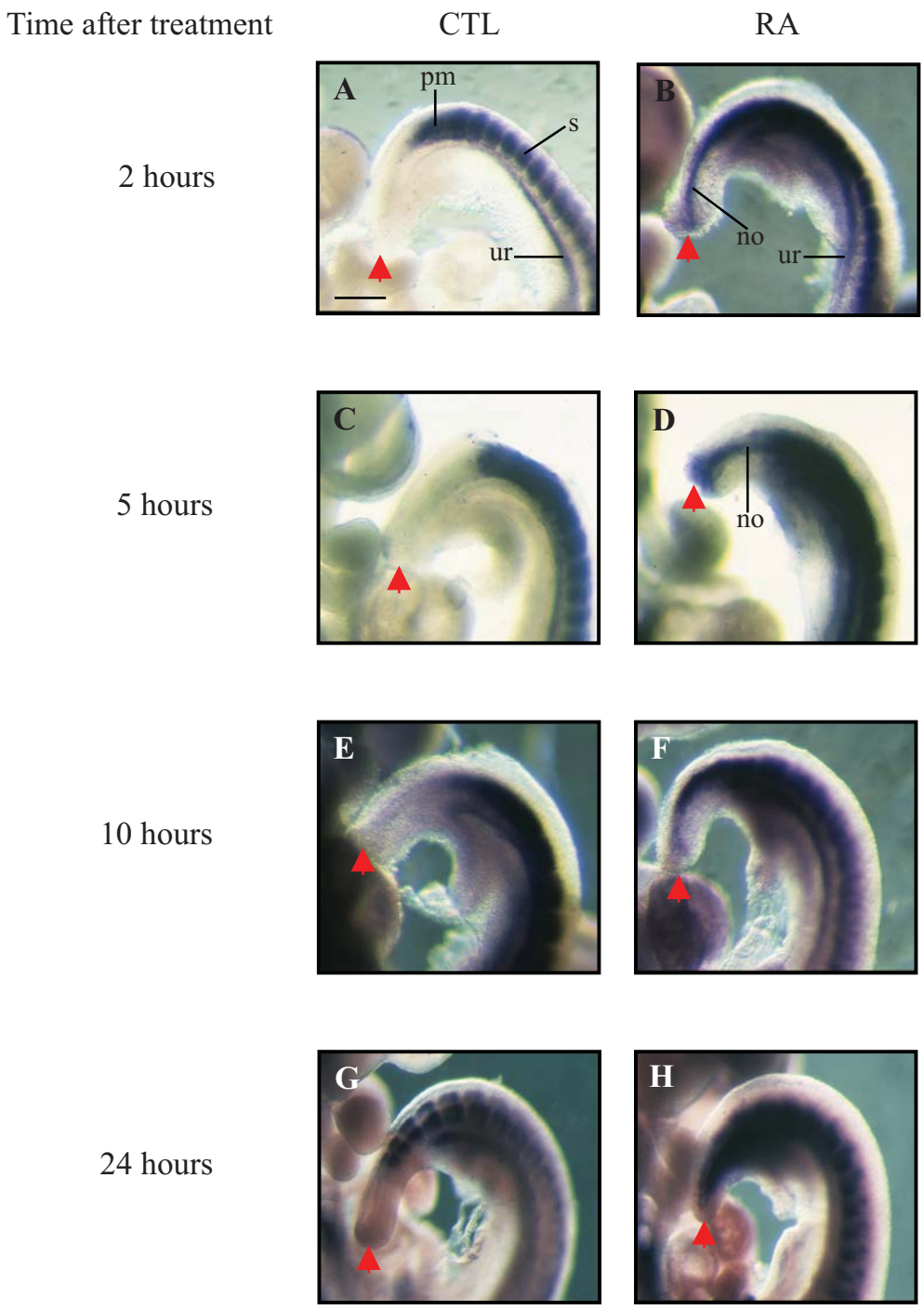
Supporting information

RA induces ArgI up-regulation and apoptosis in tail buds of mouse embryos

To determine the expression patterns of arginase I RNA and the corresponding occurrence of apoptosis in RA-stimulated mouse tail bud, whole mount *in situ* hybridization and apoptotic DNA fragmentation (DNA gel electrophoresis) were performed, respectively. A dose of 100 mg/kg body weight RA, suspended in olive oil, was injected intraperitoneally into pregnant ICR female mice at E9.5. Control mice were received equal volume of injection of olive oil only. For *in situ* hybridization experiments, embryos were explanted at 2 h (21-22 somite stage), 5 h (23-24 somite stage), 10 h (26-27 somite stage) and 24 h (33-34 somite stage) after RA administration, and were fixed immediately in freshly-prepared 4% paraformaldehyde at 4^{o}C overnight until performing *in situ* hybridization experiments. For the detection of apoptotic DNA fragmentation by DNA gel electrophoresis, embryos were explanted in ice-cold PBS either at 0 h or 6 h intervals up to 48 h after RA injection. Control embryos were received the same volume of olive oil and were collected only at the 24 h time point. The caudal end of the tail buds was removed and about fifty-sixty tails buds were collected for each time point. DNA was extracted from the tail buds, and labeled with α^{32}P-ddATP by terminal deoxynucleotidyl transferase (Tdt). Labeled DNA was then electrophoresed with 2 % agarose gel

with labeled 1 kb DNA ladder. The gel was dried and exposed to Hyperfilm-MP file for either 30 min or 2 h.

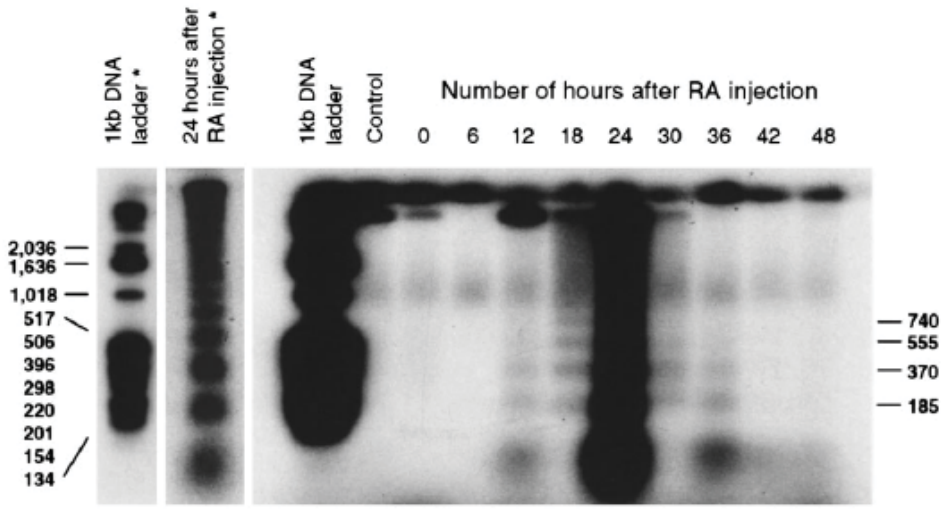
In the *in situ* hybridization experiment, it was observed that RA-stimulated arginase I expression in the caudal region of tail buds at 2 h, 5 h and 10 h (*supp* Fig. 1B, D and F) but there was no expression in 24 h (*supp* Fig. 1H). However, arginase I expression was not induced in the tail buds in the control embryos throughout the experiments (*supp* Fig. 1A, C, E and G). In addition, in both control and RA-treated embryos, arginase I was strongly expressed in the notochord in all the time points tested. To look at the expression pattern of arginase I transcript in control and RA-treated tail buds, at 2 h RA-stimulation, arginase I expression was already detected in the tail buds (*supp* Fig. 1B) but no arginase I expression was observed in tail buds of control group (*supp* Fig. 1A), and arginase I was highly expressed in the notochord in both group (*supp* Fig. 1A and B). At 5 h RA stimulation, arginase I expression was more enhanced and was the highest among the time points investigated (*supp* Fig. 1D), but no arginase I expression was observed in control tail buds (*supp* Fig. 1C). Arginase I expression was still highly expressed in the notochord of both groups (*supp* Fig. 1C and D). At 10 h RA incubation, arginase I expression was slightly down-regulated when compared with that in 5 h-stimulation (*supp* Fig. 1F), and arginase I expression was continuously expressed in the notochord of both groups (*supp* Fig. 1E and F). At 24 h RA induction, arginase I expression was completely turned off in the tail buds but still strongly expressed in notochord (*supp* Fig. 1H), which had also been observed in control groups (*supp* Fig. 1G).



Supp Fig. 1: Whole amount *in situ* hybridization of arginase in the tail bud of control and RA-stimulated embryos. A dose of 100 mg/kg RA was injected into pregnant mice for 2, 5, 10 and 24 h, and embryos were explanted, fixed and

processed for *in situ* hybridization A, C, E and G: CTL; B, D, F and K: 100 mg/kg RA. A and B: 2 h; C and D: 5 h; E and F: 10 h; G and H: 24 h. Abbreviation: no, notochord; pm, paraxial mesoderm; s, somite; ur, urogenital ridge; arrowhead, tail bud. Scale bar: 0.50 mm.

To examine the effect of RA on apoptosis in tail buds of mouse embryos, DNA was extracted for gel electrophoresis analysis of any DNA fragmentation, which is a common morphology in apoptotic cells. In the experiment, there was no DNA laddering pattern in 0 h and 6 h after RA administration but it was started to observe DNA fragmentation at 12 h. At 18 h, the intensity of DNA laddering pattern was enhanced and at 24 h, the DNA laddering pattern was strongest among the time points examined, indicating that a massive apoptotic cell death in the tail buds was processing at this stage. However, the laddering pattern was decreased after 30 h RA stimulation and at 42 h, laddering pattern was not observed, indicating that the apoptotic event was stopped at this stage. In addition, there was no DNA fragmentation in 24 h control group of tail buds, showing that the apoptotic cell death was not a common phenomenon in developing tail buds and that the apoptosis in the tail buds was completely induced by the presence of RA (*supp* Fig. 2).



Supp Fig. 2: Apoptotic DNA fragmentation of embryonic tail buds at different time points after RA administration in pregnant mice. DNA was extracted from tail buds of embryos, labeled with α^{32}P-ddATP, electrophoresed on 2 % agarose gel and analyzed by autoradiography. The exposure time was 2 h except for first two lanes (asterisks) which were exposed for 30 min.

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