TP508 Preserves Chondrocyte Cell Viability by Blocking

Apoptosis via an NO-dependent Pathway

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TP508 PRESERVES CHONDROCYTE CELL VIABILITY BY

BLOCKING APOPTOSIS VIA AN NO-DEPENDENT PATHWAY

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To my dear mother, Enling Li.

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Chapter 1 Introduction

1.1 Bone Fracture Healing and Growth Plate Model

Bone fracture healing involves a complicated network of biological responses. The healing process can be divided into five major stages: 1. the formation of initial haematoma and inflammatory responses; 2. intramembranous bone formation; 3. angiogenesis and chondrogenesis; 4. endochondral bone formation on chondrocyte templates and 5. bone remodeling (Einhorn, 1998; Simmons, 1985). During endochondral bone formation, a cartilaginous callus is formed around the facture site. The chondrocytes then become hypertrophic, and calcify their matrix. Ossification follows by replacing these calcified cartilages with bone tissues. This process mimics normal endochondral bone formation in the growth plate both histologically and biochemically (Rosier et al., 1998; Bostrom, 1998; Vortkamp et al., 1998). Therefore, the growth plate can serve as a model to mimic an important part of the bone fracture healing process.

In our lab, a rat costochondral growth plate model has been used to investigate the different factors involved in the regulation of proliferation, differentiation and matrix production of the growth plate chondrocytes. We are able to separate the resting zone chondrocytes from the prehypertrophic and hypertrophic chondrocytes

(the growth zone). Chondrocytes from both zones differ in size and shape and are regulated by different factors (Boyan et al., 1988b; Boyan et al., 1988a; Schwartz et al., 1988). For example, resting zone chondrocytes respond to the vitamin D metabolite $24,25(OH)_2D_3$ while the growth zone chondrocytes respond to $1,25(OH)_2D_3$. Both of these two vitamin D metabolites are produced actively in the growth plate and their production is regulated by transforming growth factor β1 (TGFβ1) in a zone-specific manner (Schwartz et al., 1992a). $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ regulate the production of prostaglandin E_2 (PGE_2) (Schwartz et al., 1992b) and the level of interleukin-1 (IL-1) (Dean et al., 1997) in these cells. They also regulate protein kinase C (PKC) activity (Sylvia et al., 1993), but use different pathways. $1,25(OH)_2D_3$ dependent activation of PKC involves activation of phospholipase A_2 (PLA_2) and arachidonic acid production (Boyan et al., 1998). $24,25(OH)_2D_3$ dependent activation of PKC involves phospholipase D (PLD) activation (Sylvia et al., 2001). Moreover, growth plate chondrocyte proliferation, differentiation and matrix production are also regulated by bone morphogenetic protein-2 (BMP2) in a zone-specific manner (Erickson et al., 1997). These studies demonstrate that the rat costochondral growth plate model can serve as a valuable *in vitro* test bed for studying many factors involved in regulation of endochondral bone formation and eventually, the bone fracture healing process. We used this model to study the effects of TP508 on chondrocytes, as described below.

1.2 TP508 and its Role in Bone Fracture Healing

TP508 is a 23-amino acid peptide derived from human prothrombin (amino acids 508~530).   It represents a part of the thrombin receptor-binding domain, but does not include any proteolytic domain.   As a result, it employs a different signaling pathway without the participation of proteinase-activated receptors (PARs) that are the classical thrombin peptide receptors.   Instead, TP508 binds to a poorly-known high-affinity receptor on fibroblasts (Glenn et al., 1988), which may be a member of the integrin β_3 family based on the existence of an RGD motif in the TP508 peptide sequence (Bar-Shavit et al., 1995).

Several studies have indicated that TP508 helps wound healing in both soft tissues and bones.   It accelerates wound healing in full-thickness excisional wounds in rats (Stiernberg et al., 2000), and the closure of dermal excisions (Norfleet et al., 2000b).   TP508 may achieve these functions by stimulating angiogenesis and promoting endothelial cell migration (Norfleet et al., 2000a).   Further investigation suggests that TP508 accelerates sprouting of blood vessels, but does not increase the number of new sprouts (Vartanian et al., 2006).

TP508 has also been tested in bone healing models.   It accelerates femoral fracture healing in a rat model (Simmons et al., 1998), helps healing of segmental bone defects (Sheller et al., 2004), and enhances bone formation in distraction osteogenesis in rabbits (Li et al., 2005b).   TP508 increases ERK1/2 and p38 activity in T cells, and thus activates cytokine release (Naldini et al., 2004).   The fact that it

also induces chemotaxis of osteoblasts and microvascular endothelial cells (Li et al., 2005a), may in part explain how TP508 is able to promote angiogenesis and bone formation.   Moreover, TP508 significantly increases expression of various growth factors, inflammatory response modifiers and genes related to angiogenesis in a rat bone fracture model, indicating TP508 modulates the bone cells to allow them to recruit blood vessels (Wang et al., 2005).

In our lab, we have used the rat costochondral growth model to study the effect of TP508 on chondrocyte proliferation and differentiation.   TP508 does not increase the proliferation of resting zone chondrocytes, but transiently decreases their differentiation, as evidenced by a decrease in alkaline phosphatase activity (Schwartz et al., 2005).   On the other hand, TP508 increases sulfate incorporation in resting zone chondrocytes, indicating increased cartilage extracellular matrix production. Taken together, these results suggest that during endochondral bone formation, TP508 retains the resting zone chondrocytes in a less differentiated state, while increasing the synthesis of their extracellular matrix.   Therefore, TP508 may also help bone healing by enlarging the original cartilage mass in the callus that will later serve as a template that ossification can occur on during endochondral bone formation.

1.3 The Significance of Chondrocyte Apoptosis

Apoptosis, or programmed cell death, is a well regulated cell-intrinsic process that results in removal of unwanted cells. For a specific cell population, apoptosis, together with cell proliferation and differentiation, tightly regulates the number of cells. If the rate of apoptosis and differentiation exceeds the rate of proliferation, this specific cell population will shrink. On the other hand, if the rate of proliferation exceeds the rate of apoptosis and differentiation, this cell population will expand. Therefore, to evaluate the effect of a factor on a specific cell population, it is worth noting that the effect of this factor on apoptosis is as important as its effect on cell proliferation and differentiation.

Growth plate chondrocytes are well-known to undergo apoptosis physiologically (Zenmyo et al., 1996). The more mature growth zone chondrocytes have been observed to be labeled by TUNEL staining (Ohyama et al., 1997; Hatori et al., 1995). Apoptosis in the growth plate is critical for the normal physiology of the tissue. Apoptosis is seen in situations where terminally differentiated chondrocytes have begun to express osteogenic markers (Roach et al., 1995). Apoptosis of terminally differentiated chondrocytes is caused in part by inorganic phosphate in a nitric oxide (NO)-dependent mechanism (Teixeira et al., 2001). It may also be dependent on regulation by 1,25(OH)2D3 based on a decrease in apoptosis in vitamin D receptor (VDR) knockout mice (Donohue and Demay, 2002). Although most of the research on growth plate chondrocyte apoptosis is confined to the more mature growth zone, in

our lab we have also found the less differentiated resting zone cells can also undergo

apoptosis under physiological stimuli (data not published).

1.4 The Role of Nitric Oxide in Cartilage Tissues

Nitric oxide (NO) is a small and uncharged compound that serves as a signaling molecule in various organs and tissues of animals. It has significant physiological roles in cardiovascular, neural and immune systems (Lowenstein and Snyder, 1992; Bredt and Snyder, 1994; Mayer and Hemmens, 1997). In vivo NO is mainly synthesized by three NO synthases (NOSs): two constitutively expressed NOSs, namely neuronal NO synthase (nNOS) and endothelial NO synthase (eNOS), and an inducible NO synthase (iNOS), which is activated by stimuli such as cytokines and lipopolysaccharides (Stadler et al., 1991). NO mainly binds a soluble guanylate cyclase (sGC), induces cyclic guanosine monophosphate (cGMP) production and subsequently activates multiple cGMP-dependent protein kinases to achieve its physiological functions (Denninger and Marletta, 1999). Alternatively, NO can also react with other proteins to generate S-nitrosothiols to elicit a broader range of biological reactions, though not all of them are beneficial to the cell (Stamler and Hausladen, 1998; Stamler, 1994).

In cartilage tissues, NO has significant roles in regulating many chondrocyte functions. In articular cartilage, NO inhibits integrin outside-in signaling pathways (Clancy et al., 1997), and induces breakdown of extracellular matrix (Amin and Abramson, 1998) as well as apoptosis (Blanco et al., 1995). NO-induced chondrocyte apoptosis is responsible for the pathological cell loss in osteoarthritis (van den Berg, 2001). In growth plate chondrocytes, NO is required for

physiological chondrocyte apoptosis (Teixeira et al., 2001). Besides regulating chondrocyte apoptosis, NO production is also important in maturation of growth plate chondrocytes (Teixeira et al., 2005). Therefore, NO serves as an extremely important regulator in both physiological and pathological forms of chondrocyte apoptosis.

1.5 Specific Aims and Experimental Design

The ***overall goal*** of this thesis was to use TP508 as a probe to investigate the signaling pathway that leads to chondrocyte apoptosis in the growth plate chondrocyte model. The ***general hypothesis*** was that TP508 maintains chondrocyte viability by preventing apoptosis via a NO-dependent mechanism. The problem was addressed by two specific aims.

Aim 1.   To examine the role of TP508 in chondrocyte viability and apoptosis.

The ***objective*** of this aim was to determine whether TP508 is able to reverse the loss of cell viability caused by apoptogens in chondrocytes, and whether it does so by preventing apoptosis. The ***hypothesis*** was that TP508 is able to shield the chondrocytes from undergoing apoptosis, so as to keep them alive. To address this problem, we first established chelerythrine, a PKC inhibitor, as an effective apoptogen in the rat growth plate chondrocyte model. The effects of TP508 on chondrocyte viability and apoptosis were subsequently assessed in chondrocytes treated with chelerythrine.

Aim 2.   To determine the effect of TP508 on NO production in chondrocytes and its relationship with chondrocyte apoptosis

The **objective** of this study was to determine the relationship between the NO production level and chondrocyte apoptosis. The **hypothesis** was that elevated NO production leads to apoptosis in chondrocyte, and TP508 prevents chondrocyte apoptosis by inhibiting NO production. To test the hypothesis, we first determined whether inhibiting NO production is sufficient to reduce chondrocyte apoptosis. Then we examined whether TP508 is capable of reducing NO production in chondrocytes.

Chapter 2 Material and Methods

2.1 Materials

The 23 amino acid synthetic thrombin peptide TP508, commercially known as Chrysalin®, comprised with amino acids 508-530 of human prothrombin (AGYKPDEGKRGDACEGDSGGPFV, molecular weight of 2311.5) was provided by OrthoLogic Corporation (Phoenix, AZ). The peptides were reconstituted in saline, aliquoted and stored in -70°C up to one month. NO synthase inhibitors N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine (L-NMMA), vinyl-L-NIO, 1400W, 2,3-diaminonaphthalene (DAN), paraformaldehyde, hydrogen peroxide and Triton X-100 were purchased from Sigma-Aldrich Co. (St. Louis, MO). Monohydrate monobasic sodium phosphate and sodium citrate were purchased from EM Science (Grand Island, NJ). Tamoxifen citrate and PKC inhibitor chelerythrine were purchased from Calbiochem (San Diego, CA). Cell Death Detection ELISA+ Kit, *In situ* Cell Death Detection Kit and DAB substrate were purchased from Roche Diagnostics Co. (Indianapolis, IN). Dulbecco's Modification of Eagle's Medium (DMEM) was purchased from Vediatech, Inc. (Herndon, VA). 0.25% Trypsin-EDTA was purchased from Invitrogen Inc. (Grand Island, NY). Fluorometric Caspase-3 Activity Assay Kit was purchased from Sigma-Aldrich Co. (St. Louis, MO). Biotrak

Protein Kinase C enzyme Assay Kit was purchased from Amersham Biosciences (Piscataway, NJ).   Pierce Macro BCA Protein Assay Reagent kit was purchased from Pierce Biotechnology (Rockford, IL).

2.2 Chondrocyte Cultures

The culture system used in this study has been previously described (Boyan et al., 1988b).   Both resting zone (RC) and growth zone (GC; or prehypertrophic and upper hypertrophic cell zone) chondrocytes were isolated from the costochondral junction of 120g female and male Sprague-Dawley rats by enzymatic digestion.   The chondrocytes were cultured in Dulbecco's modified Eagle medium (DMEM) containing 50μM ascorbic acid (vitamin C) and 10% fetal bovine serum (FBS) at a 5% CO_2 and 100% humidity atmosphere.   Fourth passage confluent cells were used in all experiments in this study.

2.3 Cell Number

At harvest, the chondrocytes were washed twice with DMEM and then released from the culture plate by 0.25% trypsin-EDTA digestion for 15 minutes.   DMEM with 10% FBS was used to terminate the digestion.   The chondrocyte suspensions were then subject to 500g centrifugation.   The supernatant was discarded, and the pellet was washed twice by phosphate-buffered saline (PBS) and suspended in saline.   The cell number was determined by a Z1 Coulter Particle Counter from Beckman Coulter (Fullerton, CA).

2.4 DNA Fragmentation Assays

DNA fragmentation was measured by two different assays. The first assay was conducted using the Cell Death Detection ELISA+ kit (Roche) following manufacturer's instructions with a few modifications. Chondrocytes were treated with apoptosis-inducing reagents for 24 hours. After harvesting the cells, they were lysed with lysis buffer provided in the kit. The cell lysates were incubated with anti-DNA-peroxidase and anti-histone-biotin to allow the apoptosis-induced DNA fragments form ELISA sandwiches, which were then immobilized to the streptavidin-coated wells. The amount of DNA fragments was determined by adding peroxidase substrate azino-diethyl-benzthiazoline sulfate (ABTS) to the wells and measuring absorbance at 405nm. The results were normalized to cell numbers.

The second assay was adapted from Grey et al. (Grey et al., 2002) with modifications. Cells were seeded in 24-well plates and grown to near confluence. The cells were labeled with ^{3}H-thymidine (0.5µCi/well) for 4 hours. The culture media, together with unincorporated ^{3}H-thymidine were discarded. After washing with cold DMEM twice, the cells were treated with effectors for 24 hours. The cells were harvested with trypsin-EDTA and collected. Then the cells were lysed with TE buffer (10mM Tris-HCl; 1mM EDTA, pH 7.4; 0.2% Triton X-100). To ensure the lysis of the cells, the samples were frozen and thawed three times. The lysates were subsequently centrifuged at 13,000g for 15 minutes. The radioactivity of both the supernatants and the pellets were measured using a scintillation counter. The

percentage of apoptotic cells was calculated by dividing the radioactivity of the supernatants (representing the fragmented DNA) by the sum of the radioactivity of the supernatants and the pellets (representing the total DNA).

2.5 TUNEL

TUNEL was conducted using the *In Situ* Cell Detection Assay kit (Roche) following manufacturer's instruction. Chondrocytes were treated with effectors for 24 hours and fixed with 4% paraformaldehyde. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide. The cells were then treated with permeabilisation solution (0.1% sodium citrate and 0.1% Triton X-100). The permeabilised cells were incubated with terminal transferase for 1 hour. After the end labeling of DNA was converted into optical signal by incubating with DAB substrate (Roche), apoptotic cells were observed using a light microscopy. Images are taken using Image Pro Plus 5.0 MediaCybernetics (Silver Spring, MD).

2.6 PKC Activity

Chondrocytes were treated different amount of TP508 for 9, 90, and 270 minutes, and 6 and 12 hours. At harvest, the cells were washed twice with 500 μl PBS, and 100 μl of RIPA buffer was added to lyse the cells. The PKC activity of each sample was measured using the Biotrak Protein Kinase C enzyme Assay Kit and procedure from Amersham Biosciences (Piscataway, NJ). The system is based upon the PKC catalyzed transfer of $[^{32}P]$-PO4 from $[^{32}P]$-γ-ATP to a peptide specific for PKC. The

reaction mixture consisted of 3 mM calcium acetate, 0.075 mg/ml lα-phosphatidyl-l-serine, 6 μg/ml phorbol 12-myristate 13-acetate, 150 μM PKC specific peptide, and 7.5 mM dithiothreitol in a 50 mM Tris-HCl buffer containing 0.05% (w/v) azide at pH 7.5. 25 μl of the mixture was added to 25 μl of each sample. γ-ATP phosphorous-32 was mixed with a 1.2 mM ATP, 30 mM Hepes, 72 mM magnesium chloride solution at pH 7.4 and a total count of $450,000 \pm 20,000$ cpm per 5 μl was obtained. 5 μl of radioactive ATP buffer was added to each sample. The samples were centrifuged and incubated at 37°C for 15 min. After incubation, 10 μl of the stop reagent containing 300 mM ortho-phosphoric acid and carmosine red was added and the samples were centrifuged again. The samples were mixed, and 35 μl was transferred to the peptide binding papers provided in the kit. The papers were dried for 5 min and then washed twice in 75 mM ortho-phosphoric acid solution with agitation for 5 min. The paper disks were transferred into scintillation vials filled with 10 ml of Ready-safe and counted for 1 min each. The total pmols phosphate (P) transferred per minute were calculated as follows: $P = (T \times 1000)/(I \times R)$, where I is the incubation time. The specific activity (R) of 1.2 mM Mg [^{32}P]ATP is equal to the total count ($450,000 \pm 20,000$ cpm) per 5 μl divided by the number of moles of ATP (6×10^{-9}) per 5 μl. The total phosphate (T) transferred to peptides and endogenous proteins is equal to the sample cpm minus the blank cpm. The blank cpm was measured using cell lysate free reactions with reaction buffer to determine the background. Each sample was then normalized by the total protein content per well.

2.7 Macro Protein Assay

All samples were normalized by total protein content using the Pierce Macro BCA Protein Assay Reagent kit from Pierce Biotechnology (Rockford, IL).   Reagents A and B were mixed in a 50:1 ratio to make the working reagent.   5 μl of each sample was aliquoted in duplicate to 96-well plates, and 200 μl of the working reagent was added to the sample plates.   They were incubated at 37°C for 30 min and read in the Bio-Rad microplate reader at 570 nm.

2.8 Cell Viability Assay

Cell viability was measured as a function of mitochondrial dehydrogenase enzyme activity using the CellTiter 96® Cytotoxicity Assay kit from Promega (Madison, WI) following the manufacturer's instruction with.   The cells were treated with or without effectors in 24-well plates for 24 hours.   After the treatment period ended, the cells were incubated with dye solution (contains tetrazolium salt, or MTT) for 4 hours in 37^oC.   Subsequently solubilization/stop solution was added and the samples were allowed to incubate overnight to lyse the cells and release the formazan crystals generated by the viable cells.   The absorbance of the formazan was measured using a microplate reader with absorbance at 570nm subtracted by that at 655nm.   The results are presented as the percentage compared with the negative control samples.

2.9 Nitric Oxide Production

NO production was evaluated by determining the concentration of accumulated nitrite in the culture media using a fluorescence assay with 2,3-diaminonaphthalene

(DAN) as originally described by Misko et al. (Misko et al., 1993) with modifications. After the cells were treated with effectors for 24 hours, 200μl of cell culture media were removed from each well and mixed with 16μl DAN assay buffer [DAN (50μg/ml) in 0.62 M HCl]. The mixtures were put in dark place for 15 minute incubation. The reaction was subsequently stopped by 20μl stopping reagent (2.8M NaOH). The fluorescence of each sample was then measured using a fluorescence plate reader, with excitation at 364nm and emission at 406nm. The fluorescence was converted into concentration of nitrite in the media using a standard curve made with sodium nitrite. The results were normalized to cell numbers.

2.10 Statistical Analysis

All experiments were conducted a minimum of two times to ensure validity of the results. For each individual experiment, the results are shown as the mean ± standard error of at least five individual cultures. Data were first analyzed by analysis of variance; when statistical differences were detected, the Student's t-test for multiple comparisons using Bonferroni's modification was used. Reliability of the repeated experiments is tested by Wilcoxon signed rank test. P-values < 0.05 were considered to be significant in every experiment.

Chapter 3 TP508 Rescues Chondrocytes Viability by Inhibition of Apoptosis

3.1 Hypothesis and Rationale

The working *hypothesis* of Aim 1 was: Chelerythrine causes apoptosis in chondrocytes; TP508 is able to counter the apoptosis caused by it. The ***rationale*** of this study is based on the following observations. Related studies in our lab show that TP508 increases both ERK1/2 and PKC activity in chondrocytes (unpublished data), and both of these signaling kinases are associated with inhibition of apoptosis in chondrocytes (Shakibaei et al., 2001; Kim et al., 2002). In preliminary experiments, we found that TP508 treatment prevented the decrease in chondrocyte cell number caused by the PKC inhibitor chelerythrine. Since TP508 does not increase the proliferation of chondrocytes (Schwartz et al., 2005), it is probable that it achieves this effect by blocking chondrocyte apoptosis.

3.2 Establishment of Chelerythrine and Tamoxifen as Apoptogens in Growth

Plate Chondrocytes

To examine the effect of chelerythrine on growth plate chondrocyte apoptosis, we treated both male and female rat costochondral cartilage resting zone and growth zone cells with chelerythrine (10μM) and used two different methods to evaluate apoptosis: measurement of DNA fragmentation and DNA nick end.   Tamoxifen, another proven PKC inhibitor (Schwartz et al., 2002), was also included in this study.

Based on DNA fragmentation following a 24 hour treatment with the apoptogens, both tamoxifen and chelerythrine caused significant dose-dependent increases in apoptosis (Figure 3-1).   The effects of the apoptogens were sex-dependent.   In chondrocytes from female animals, chelerythrine and tamoxifen had a similar effect. However, in chondrocytes from male animals, tamoxifen caused a significantly smaller increase in apoptosis than chelerythrine.   In both cases the effects of chelerythrine and tamoxifen were additive.
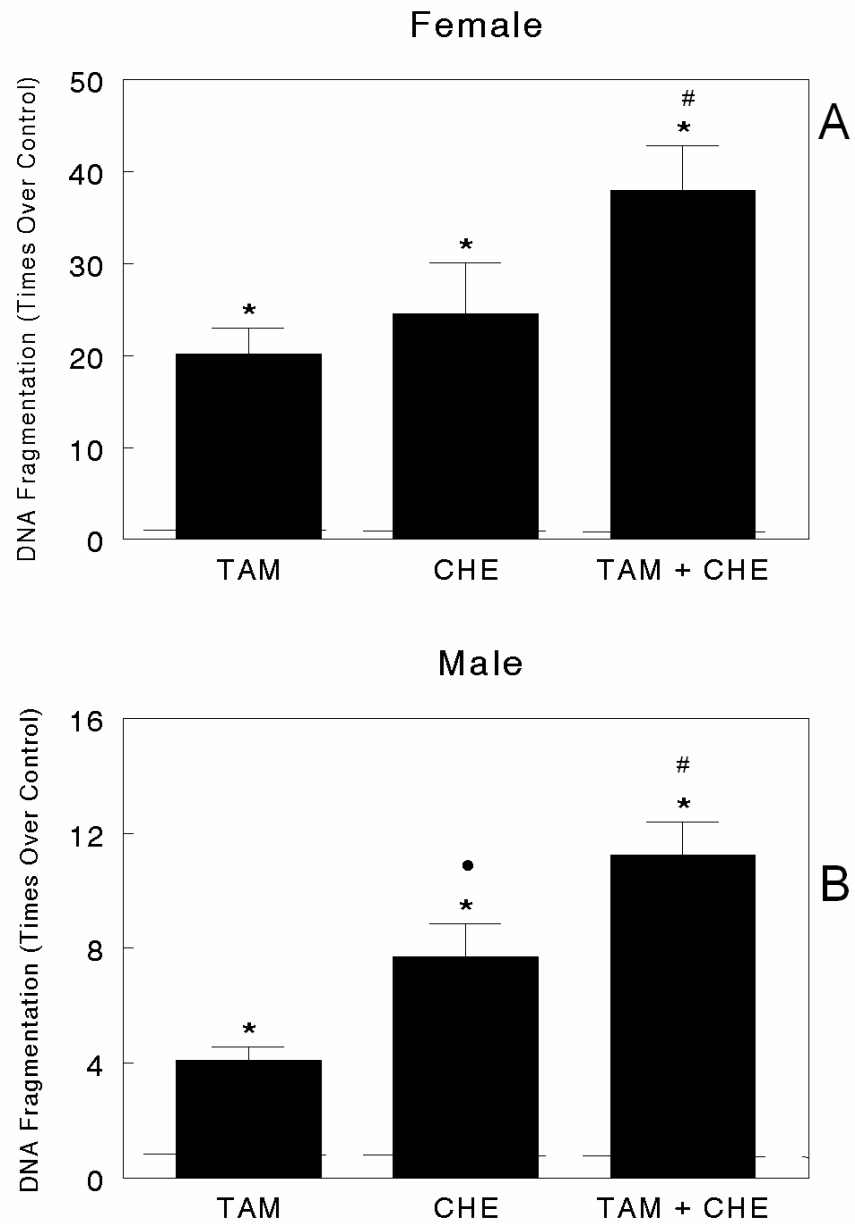
Figure 3-1 DNA fragmentation ELISA for cells of confluent resting zone costochondral growth plate chondrocyte cultures isolated from female (A) and male (B) rats. Cultures were treated with 10^{-9}M tamoxifen (Tam) and/or 10μM chelerythrine (Chel) for 24 hours. Data are means ± SEM for N = 6 independent cultures. Data presented are from one of at least two separate experiments, all of which had comparable results. *: $p<0.05$ against control; • : $p<0.05$ against tamoxifen treatment; #: $p<0.05$ against chelerythrine treatment.

These observations were confirmed by TUNEL assay of fixed cells (Figure 3-2). In the control sample, there were few cells undergoing apoptosis, while it was obvious that both chelerythrine and tamoxifen both caused significant apoptosis after 24 hours. It was hard to judge which of these two caused more apoptosis, however, since both seemed to saturate the cells examined with apoptosis. This was also true for cells treated with chelerythrine and tamoxifen in combination.

Control   10µM Chelerythrine

10^{-9}M Tamoxifen 10µM Chelerythrine + 10^{-9}M Tamoxifen
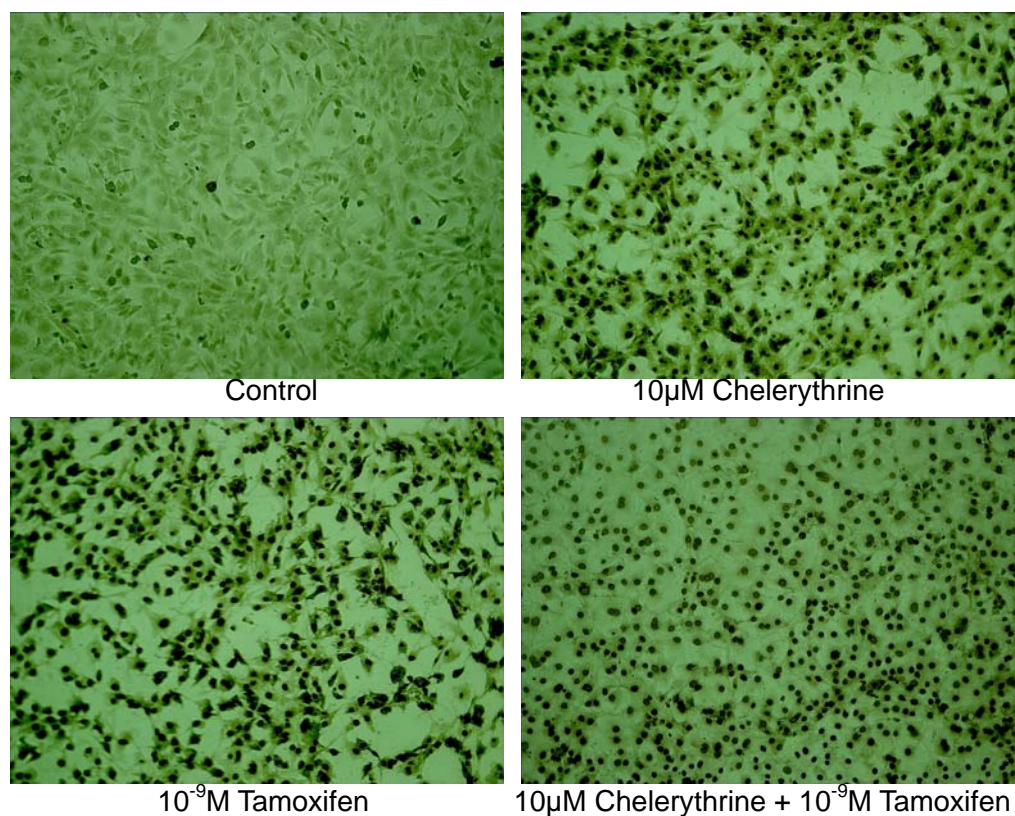
Figure 3-2 Effect of chelerythrine and tamoxifen on the number of TUNEL-positive cells of confluent resting zone costochondral growth plate chondrocyte cultures isolated from female rats.   Cultures were treated with 10-9M tamoxifen, 10µM chelerythrine or their combination for 24 hours. Data presented are from one of at least two separate experiments, all of which had comparable results.

Based on the DNA fragmentation ELISA and TUNEL results, both tamoxifen and chelerythrine were shown to be bona fide inducers of chondrocyte apoptosis. These results indicate that inhibition of PKC may cause apoptosis in chondrocytes. Chelerythrine were used in subsequent studies to induce apoptosis in growth plate chondrocytes, and test whether TP508 was able to reverse its effect.
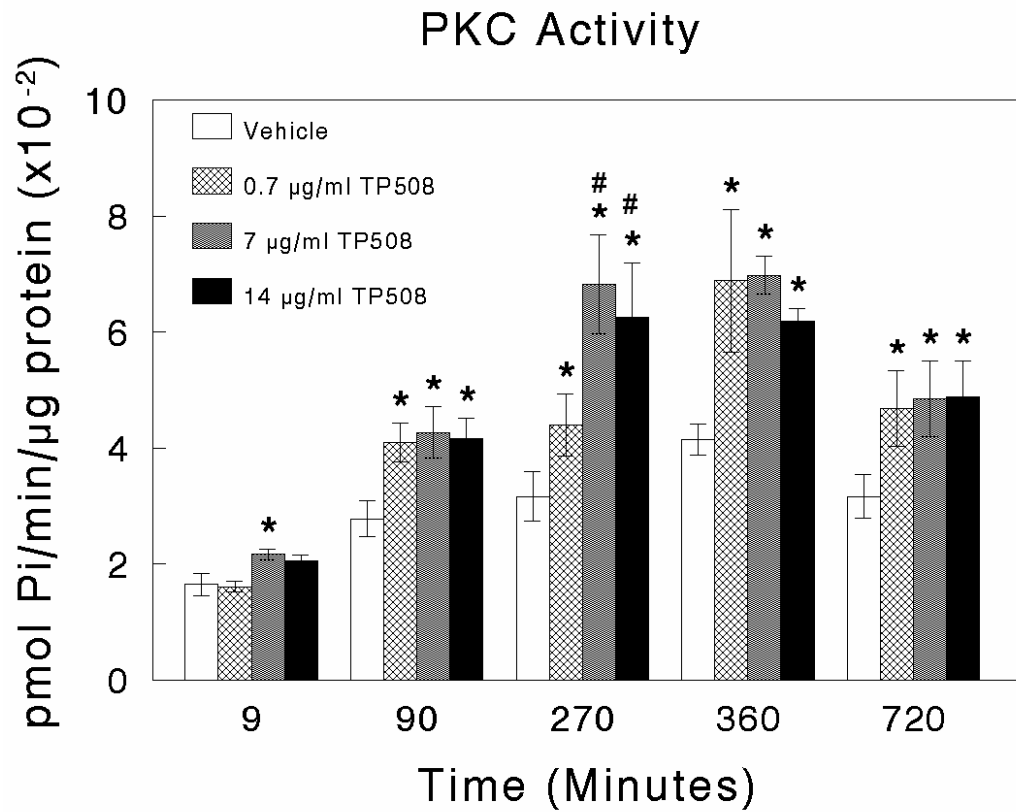
Figure 3-3 Effect of TP508 on PKC specific activity of confluent resting zone costochondral growth plate chondrocyte cultures isolated from female rats. Cultures were treated with 0.7 to 14μg/ml TP508 for 9 to 720 minutes. Data are means ± SEM for N = 6 independent cultures. Data presented are from one of at least two separate experiments, all of which had comparable results. *: P<0.05 vs. vehicle groups; #: P<0.05 vs. 0.7μg/ml TP508 groups.

3.3 TP508 Recovers Chondrocyte Cell Viability Loss Caused by

Chelerythrine by Reducing Apoptosis

TP508 activated PKC in resting zone cells in a time-dependent manner (Figure 3-3). The stimulatory effect was evident at 90 minutes and activity in the treated cultures remained elevated through 360 minutes. By 12 hours, PKC activity dropped, but it did not return to the base line level. Chelerythrine is a well-established PKC-specific inhibitor (Herbert et al., 1990). This suggested the possibility that chelerythrine induced apoptosis in growth plate chondrocytes by decreasing PKC activity. The fact that TP508 was able to increase PKC activity in the same cell type indicated that it might be able to antagonize the effect of chelerythrine.
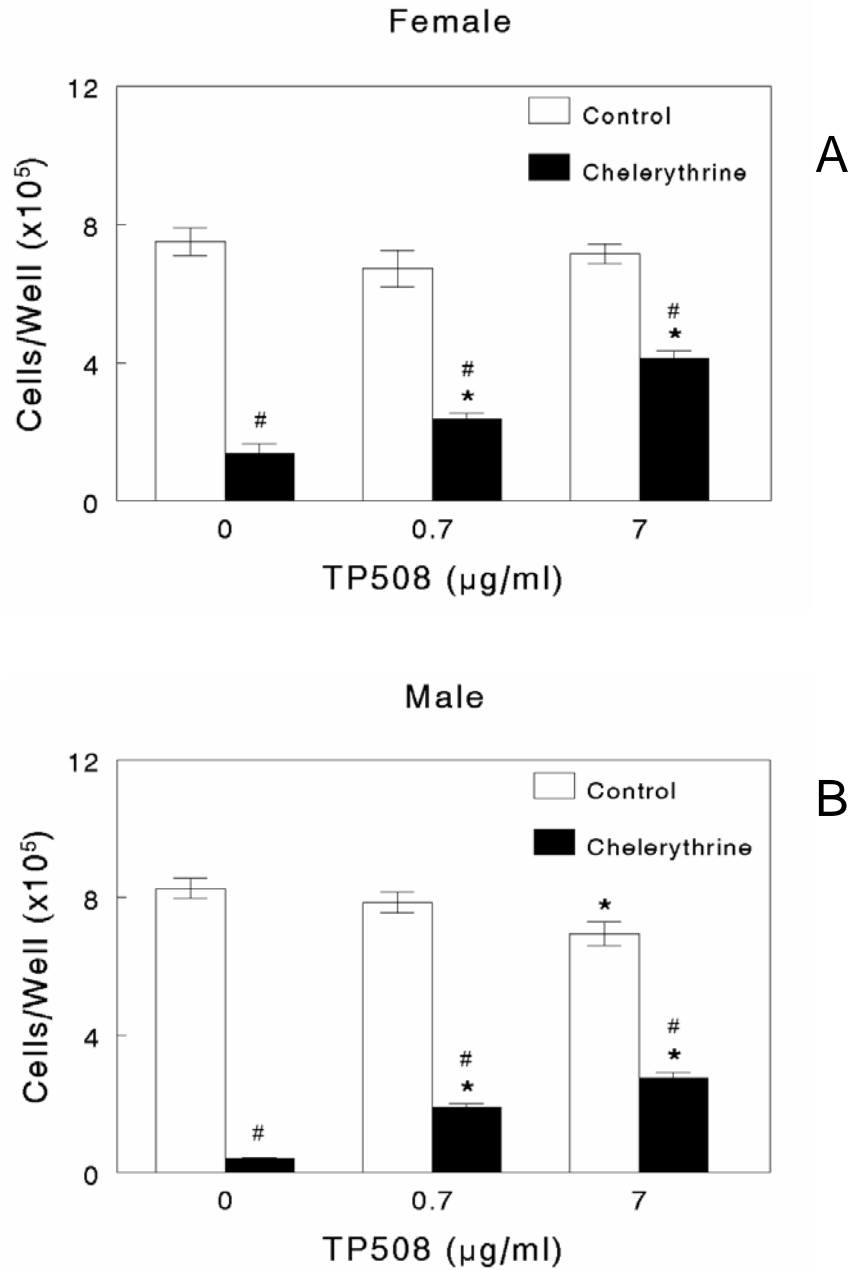
Figure 3-4 Effect of chelerythrine on cell number of confluent resting zone costochondral growth plate chondrocyte culture isolated from female (A) and male (B) rats. Cultures were treated with 10μM chelerythrine with or without 0.7 or 7μg/ml TP508 for 24 hours. Data are means ± SEM for N = 6 independent cultures. Data presented are from one of at least two separate experiments, all of which had comparable results. *: p<0.05 against respective groups without TP508 treatment; #: p<0.05 against respective groups with the same amount of TP508 treatment.

When resting zone chondrocytes were treated with 10μM chelerythrine, there was a significant loss of cell number (Figure 3-4).   In female chondrocytes, chelerythrine caused a 72% decrease in cell number, while in male chondrocytes, the cell number decreased almost 95%.   However, when TP508 was added together with chelerythrine, the effect of the PKC inhibitor on cell number was reduced and this was dependent on the concentration of TP508.   When control cultures of resting chondrocytes were treated with both chelerythrine and 7μg/ml TP508, cell number recovered by 33% and 55% in male and female cells, respectively.

Similar results were found when assessing the effects of chelerythrine and TP508 on metabolic activity of the cells (Figure 3-5).   Treatment of female resting zone chondrocytes with 10μM chelerythrine for 24 hours resulted in a 94% decrease in cell viability.   TP508 caused a dose-dependent recovery of cell viability in cells treated with chelerythrine.   When 7μg/ml TP508 was added with chelerythrine, activity was increased to 32% of control cultures.   These results confirmed that TP508 blocked the effects of chelerythrine on cell viability.
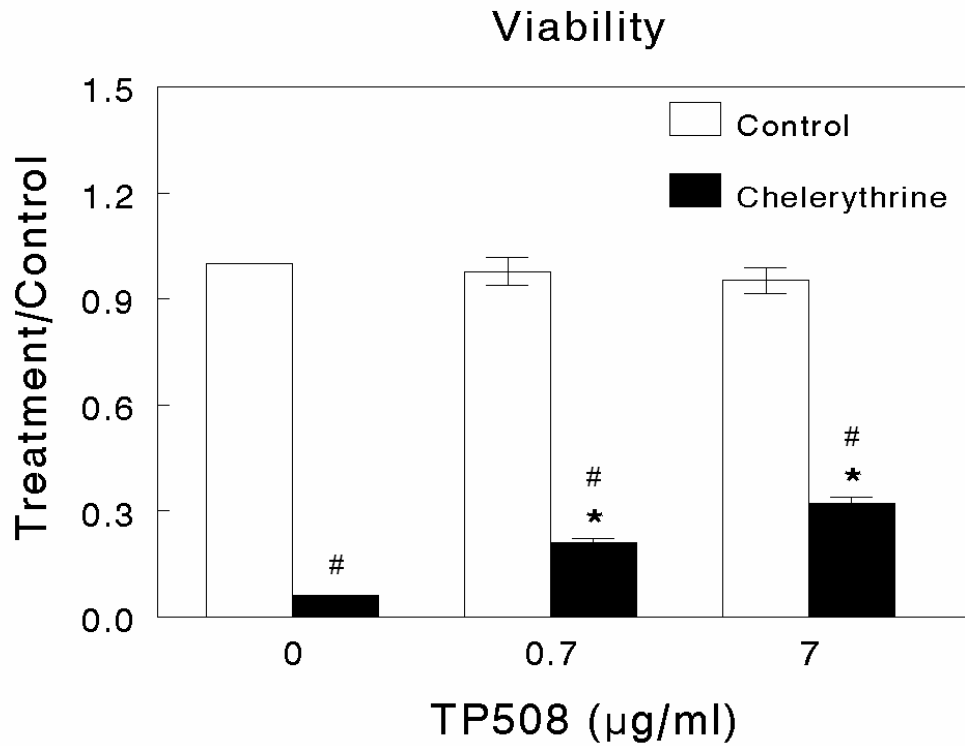
Figure 3-5 Effect of chelerythrine on cell viability of confluent resting zone costochondral growth plate chondrocyte culture isolated from female rats. Cultures were treated with 10μM chelerythrine with or without 0.7 or 7μg/ml TP508 for 24 hours.   Data are means ± SEM for N = 6 independent cultures.   Data presented are from one of at least two separate experiments, all of which had comparable results.   *: $p < 0.05$ against respective groups without TP508 treatment; #: $p < 0.05$ against respective groups with the same amount of TP508 treatment.

There are two factors affecting the total number of viable cells in a given population. One is cell proliferation, while the other is cell death. When interpreting the data from an MTT assay, both cell proliferation and cell death should be considered. A lower number of viable cells may be caused by either elevated cell death or decreased cell proliferation. In the case of TP508 treatment, the recovery of the number of viable cells can either be interpreted as that TP508 inhibited cell death caused by chelerythrine, or itself caused an increase of chondrocyte proliferation, or a combination of both effects. However, TP508 did not increase resting zone chondrocyte proliferation in a previous study (Schwartz et al., 2005), and this was confirmed by both the cell number experiment and the MTT experiment, in both of which TP508 treatment alone did not cause an increase in either cell number or cell viability, but actually caused a slight decrease in both cases when 7μg/ml TP508 was applied to the resting zone chondrocytes. Therefore, it was unlikely that TP508 recovered chondrocyte viability by directly increasing their proliferation. This indicated that TP508 probably achieved this effect by inhibiting cell death caused by chelerythrine.
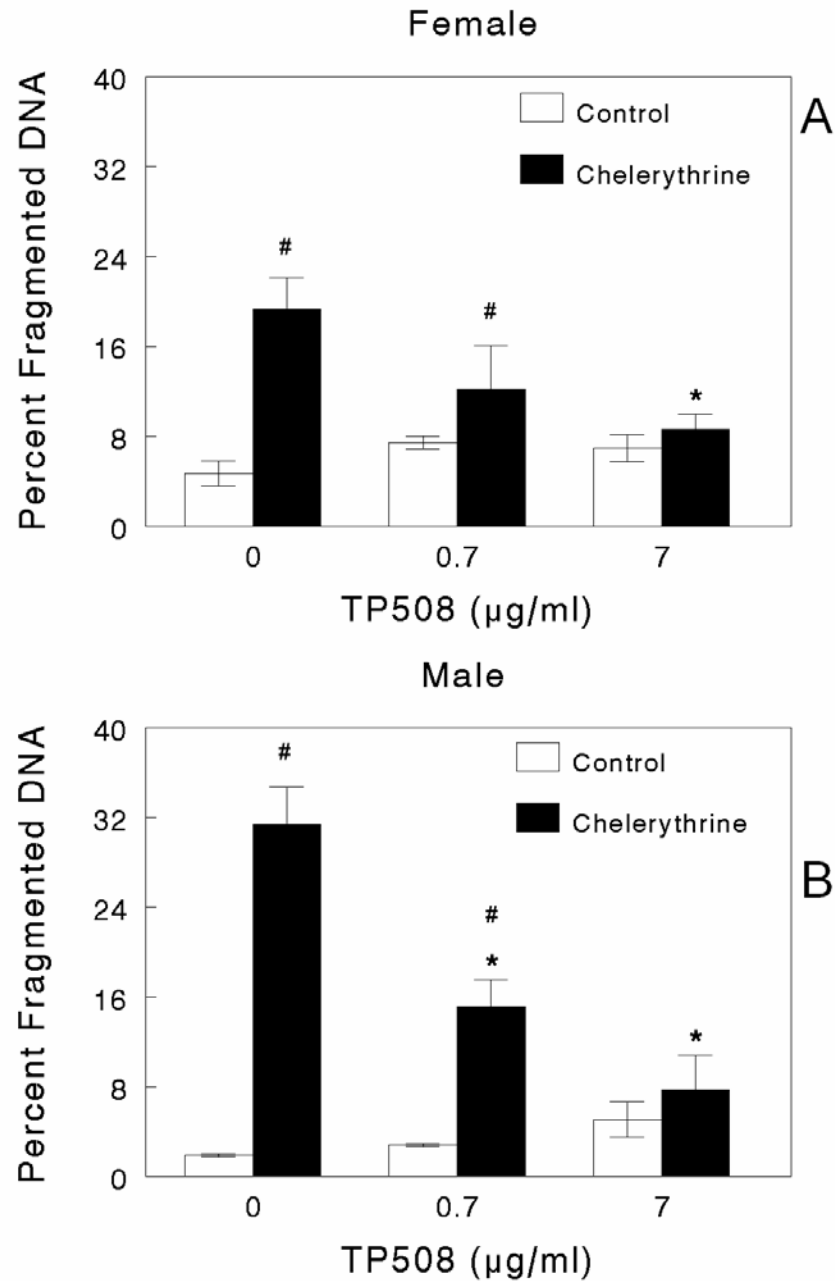
Figure 3-6 Effect of chelerythrine on DNA fragmentation of confluent resting zone costochondral growth plate chondrocyte culture isolated from female (A) and male (B) rats. Cultures were treated with 10μM chelerythrine with or without 0.7 or 7μg/ml TP508 for 24hours. Data are means ± SEM for N = 6 independent cultures. Data presented are from one of at least two separate experiments, all of which had comparable results. *: p<0.05 against respective groups without TP508 treatment; #: p<0.05 against respect groups with the same amount of TP508 treatment.

Measurement of DNA fragmentation using the radioactive DNA fragmentation assay confirmed our previous observations using DNA fragmentation ELISA and TUNEL that chelerythrine caused a significant increase in apoptosis in both male and female chondrocytes (Figure 3-1, 3-2).   Using this assay, we found that TP508 caused a dose-dependent inhibition of apoptosis induced by chelerythrine in both male and female cells.   When treated with 7μg/ml TP508, chondrocyte apoptosis was actually restored to the base line level.   This differed from the results based on MTT assay, which showed that TP508 only partially recovered the cell viability loss caused by chelerythrine.

There are two explanations for this discrepancy.   First, apoptosis is a quick process, and not all the cells undergo apoptosis at the same time when subjected to apoptogen assault.   It is quite possible that after 24 hours the level of apoptosis caused by chelerythrine was restored to control level by TP508, but many of the cells had already been lost by apoptosis by then and left no trace in the cell population. The other possible explanation is that part of the cell death caused by chelerythrine was by necrosis, which always accompanies apoptosis.   In any case, these experiments showed clear evidence that at least part of TP508's effect on recovery of cell viability loss caused by chelerythrine was through inhibiting apoptosis.

Chapter 4 TP508 Inhibits Chondrocyte Apoptosis by Blocking NO Production

4.1 Hypothesis and Rationale

The working *hypothesis* of this aim was that the inhibitory effect of TP508 on chondrocyte apoptosis is via reducing NO production. The *rationale* was that elevated NO production is related to both physiological and pathological apoptosis of chondrocytes (van den Berg, 2001; Teixeira et al., 2001). The NO donor SNOG induces chondrocyte apoptosis (Teixeira et al., 2001). NO enhances maturation of chondrocytes (Schwartz et al., 2005; Teixeira et al., 2001; Teixeira et al., 2005), while TP508 works against it (Schwartz et al., 2005; Teixeira et al., 2001; Teixeira et al., 2005). Thus, TP508 may elicit its effect by blocking NO production, and in so doing, it may also block chondrocyte apoptosis.
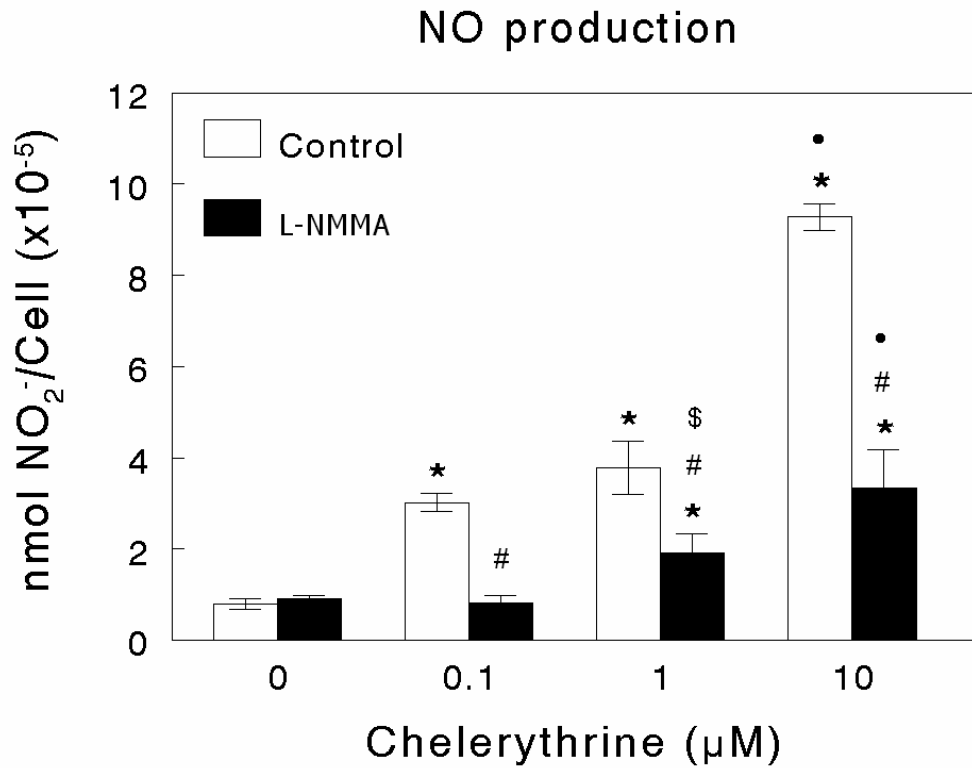
Figure 4-1 Effect of L-NMMA on chelerythrine-induced NO production of confluent resting zone costochondral growth plate chondrocyte cultures isolated from female rats. Cultures were treated with 0.1 to 10μM chelerythrine in the presence or absence of 7μg/ml TP508 for 24 hours. Data are means ± SEM for N = 6 independent cultures. Data presented are from one of at least two separate experiments, all of which had comparable results. *: P<0.05 vs. 0μM chelerythrine groups; #: P<0.05 vs. control groups; $: P<0.05 vs. 0.1μM chelerhthrine; ·: P<0.05 vs. 1μM chelerythrine groups.

4.2 Inhibition of NO Production Reduces Chondrocyte Apoptosis

Chelerythrine caused a dose-dependent increase in NO production in 24 hours (Figure 4-1). This increase was observed at concentrations as low as 0.1μM. 10μM chelerythrine caused about 11 times increase of NO production compared with the control group. The universal NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) alone did not cause any significant change in NO production compared with the control cultures. However, L-NMMA significantly decreased NO production caused by chelerythrine treatment at every concentration examined. These results showed that L-NMMA was an effective NO production inhibitor in this resting zone chondrocyte culture system.
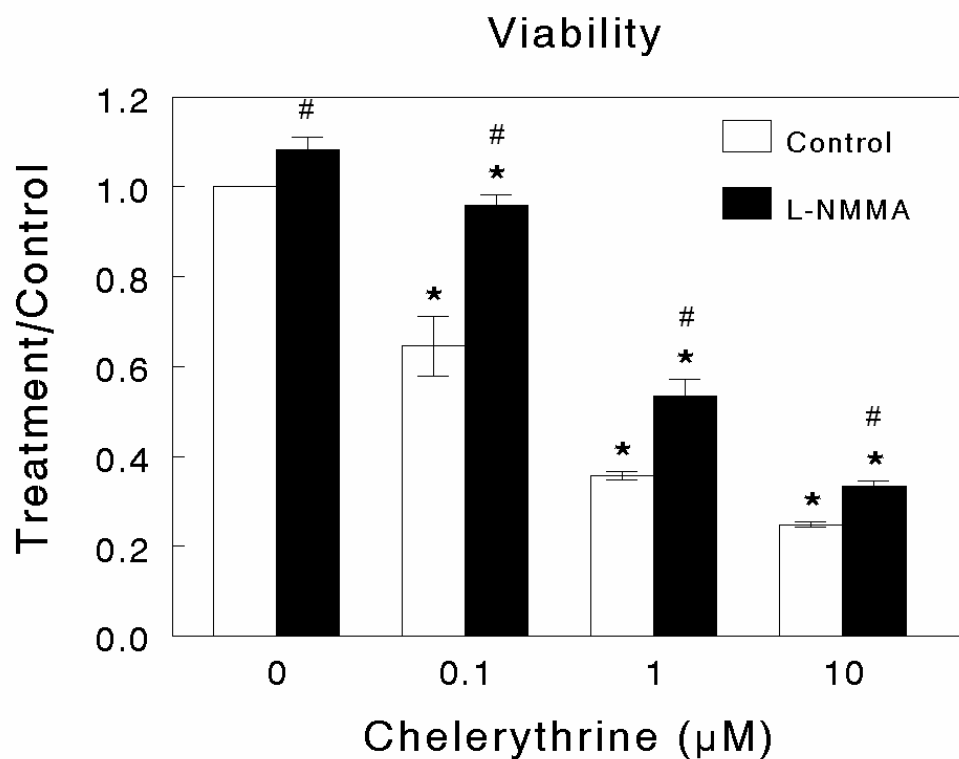
Figure 4-2 Effect of L-NMMA on chelerythrine-induced cell death of confluent resting zone costochondral growth plate chondrocyte cultures isolated from female rats. Cultures were treated with 0.1 to 10μM chelerythrine in the presence or absence of 7μg/ml TP508 for 24 hours. Data are means ± SEM for N = 6 independent cultures. Data presented are from one of at least two separate experiments, all of which had comparable results. *: P<0.05 vs. 0μM chelerythrine groups; #: P<0.05 vs. control groups.

Inhibition of NO production also reduced the effects of chelerythrine on MTT activity (Figure 4-2). Chelerythrine decreased the cell viability of resting zone chondrocytes in a dose-dependent manner. 10μM chelerythrine caused the cell viability of resting zone chondrocytes drop to 33% compared with control. L-NMMA is able to partially reverse the drop in cell viability caused by chelerythrine in all concentrations. The patterns that L-NMMA decreased chelerythrine-caused cell death were consistent with the patterns that L-NMMA decreased NO production caused by chelerythrine.

The results of these experiments indicate that in resting zone chondrocytes, NO production is associated with increased cell death. Higher NO production resulted in reduced viability of the cells. Moreover, blocking NO production by a universal NO synthase inhibitor reversed cell death caused by an established chondrocyte apoptosis inducer.
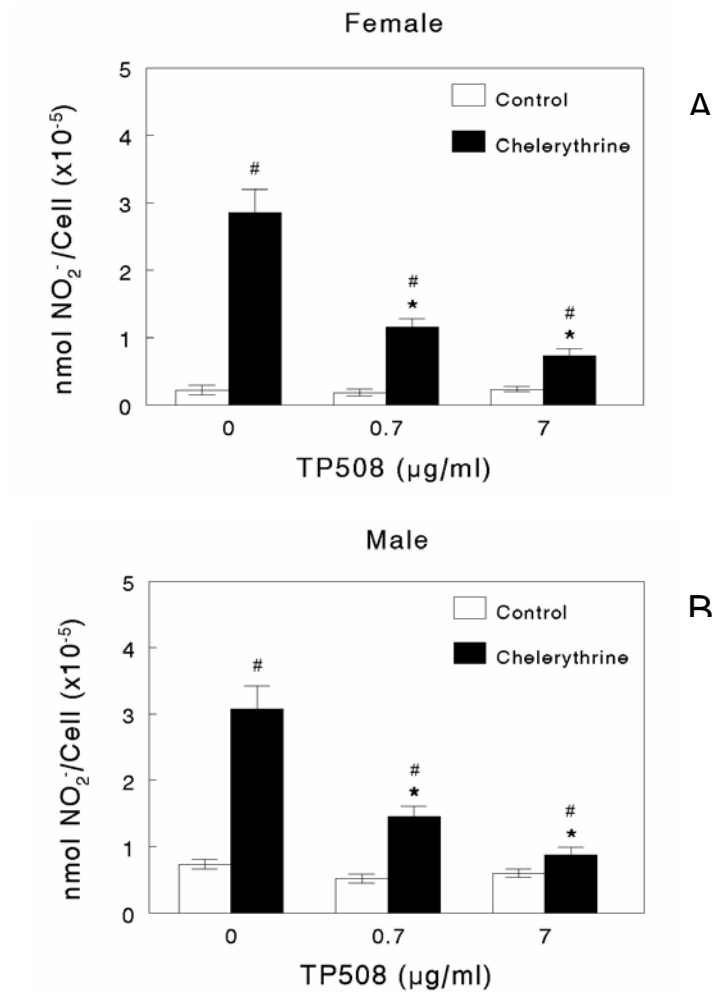
Figure 4-3 Effect of chelerythrine on NO production of confluent resting zone costochondral growth plate chondrocyte culture isolated from female (A) and male (B) rats. Cultures were treated with 10μM chelerythrine with or without 0.7 or 7μg/ml TP508 for 24 hours. Data are means ± SEM for N = 6 independent cultures. Data presented are from one of at least two separate experiments, all of which had comparable results. *: p<0.05 against respective groups without TP508 treatment; #: p<0.05 against respective groups with the same amount of TP508 treatment.

4.3 TP508 Inhibits NO Production in Resting Zone Chondrocytes

TP508 also inhibited NO production induced by chelerythrine (Figure 4-3). When female resting zone cells were treated with 10μM chelerythrine in the presence of TP508 for 24 hours, there was a significant increase in NO production in female resting zone chondrocytes, as indicated in previous experiments. The male cells showed similar response to chelerythrine as the female cells. In both sexes TP508 caused a dose-dependent inhibition of NO production. When 7μg/ml TP508 was added with chelerythrine together to the chondrocytes, NO production was reduced to almost a third compared to chelerythrine alone. TP508 did not significantly change the background NO production by the control cells, indicating that its function was mainly blocking NO production caused by chelerythrine. This was consistent with the previous finding that TP508 did not significantly increase the viability of the control cells but acted to block apoptosis caused by chelerythrine. This study also showed that both male and female chondrocytes responded to both chelerythrine and TP508 in a similar pattern, which was also consistent with previous study on cell number and apoptosis.

To determine the time point that TP508 started to block NO production caused by chelerythrine, we performed a time course study. In this study, chondrocytes were treated with chelerythrine with or without TP508 for 9, 90, 270, 720 and 1440 minutes, after which NO production was measured.
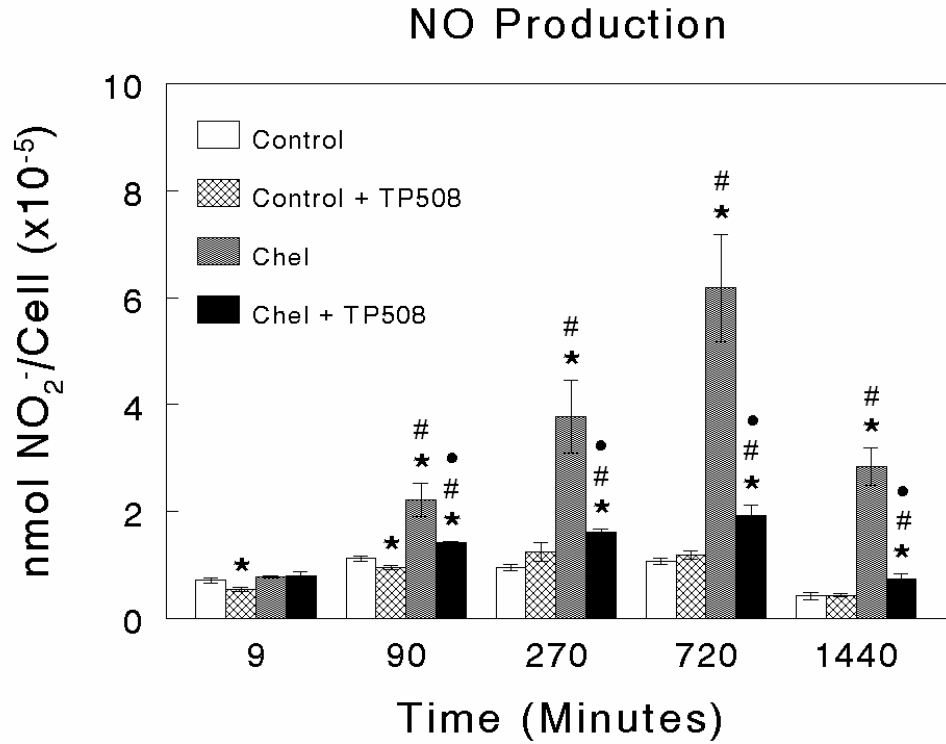
Figure 4-4 Effect of TP508 on chelerythrine-induced NO production of confluent resting zone costochondral growth plate chondrocyte cultures isolated from female rats.   Cultures were treated with 7μg/ml TP508, 10μM chelerythrine, or their combination for 9 to 1440 minutes.   Data are means ± SEM for N = 6 independent cultures.   Data presented are from one of at least two separate experiments, all of which had comparable results.   *: P<0.05 vs. control groups; #: P<0.05 vs. 7μg/ml TP508 groups; ·: P<0.05 vs. 10μM chelerythrine groups.

The inhibitory effect of TP508 on NO production was rapid and was evident in control cultures within 9 minutes (Figure 4-4). The increase in NO seen in female resting zone cells treated with chelerythrine was seen at 90 minutes and was effectively blocked by TP508 at that time. NO production in the chelerythrine treated cultures continued to increase with time and was still significantly elevated after 12 hours of treatment. By 270 minutes, the inhibitory effect of TP508 was no longer evident in control cultures, but it was sufficient to block more than 90% of the NO produced in response to chelerythrine.

Chapter 5 Conclusions and Discussion

The results of this study have demonstrated that TP508 may regulate the survival signal in the resting zone chondrocytes of the growth plate. TP508 treatment significantly rescues resting zone chondrocytes from apoptosis induced by chelerythrine, an apoptogen in this type of cells, although TP508 does not affect the background apoptosis level of resting zone chondrocytes. TP508 apparently stimulates these chondrocytes to survive at least partially by lowering the NO production caused by those apoptogens. Taken together, it indicates that TP508 shields the resting zone chondrocytes from cell death.

This result, as well as previous finding that TP508 retains resting zone chondrocytes in less differentiated phenotype and expands the cartilage mass (Schwartz et al., 2005), indicates that the function of TP508 in the cartilage callus is to preserve the resting zone chondrocytes, both temporally and spatially in the initial build-up stage of endochondral bone formation. By doing so, TP508 allows more cartilage to form, and subsequently more bone also forms on this cartilage template, so as to improve bone healing.

PKC activation has been associated with proliferation in many cell types (Zhou et

al., 1993; Carlin et al., 1999; Piacentini et al., 2000).   In contrast, inhibition of PKC is associated with apoptosis. The PKC inhibitor chelerythrine induces apoptosis in multiple types of cells (Platzbecker et al., 2003; Yamamoto et al., 2001; Lewis et al., 2003).   Another PKC inhibitor, straurosporine, has been widely used as a positive control in apoptosis studies.   Therefore, inhibition of PKC may lead to apoptosis in resting zone chondrocytes.   In this study, chelerythrine significantly induces apoptosis in resting zone chondrocytes, as judged by DNA fragmentation and TUNEL staining.   Since TP508 increases PKC activity in resting zone chondrocytes, it may assert its anti-apoptotic function by an antagonistic effect on PKC activity against chelerythrine.   However, chelerythrine can also induce apoptosis in a PKC-independent pathway (Yu et al., 2000), and at least in some cells tamoxifen's apoptotic function is via estrogen receptors and may be PKC-independent. Therefore, further research is required to settle this issue.   It has been suggested that chelerythrine may also directly inhibit some bcl-2 family proteins (Chan et al., 2003). Since TP508 regulates the expression of many genes via a membrane-receptor pathway (Wang et al., 2005), we plan to investigate whether TP508 also works through a membrane of bcl-2 family: either it is upregulating a pro-apoptotic member or downregulating an anti-apoptotic member of bcl-2 family.

NO production has been correlated with both physiological apoptosis of growth plate chondrocytes and pathological apoptosis of articular chondrocytes (van den Berg, 2001; Teixeira et al., 2001).   Blocking PKC activity by chelerythrine induces NO production in platelets (Freedman et al., 2000).   A similar mechanism can be at work

in resting zone chondrocytes.   NO production is carried out mainly by three types of NO synthase: eNOS, nNOS and iNOS.   All of them are expressed in growth plate (Teixeira et al., 2005; Hukkanen et al., 1999; van den Berg, 2001).   From our current study, it is likely that chelerythrine mainly causes NO production via iNOS activation, and TP508 blocks the same pathway.   nNOS does not appear to be involved in this process.   However, an involvement of eNOS cannot be ruled out, since we are not able to find a specific eNOS inhibitor.   Further research should be focused on the effect of chelerythrine and TP508 on an eNOS-knockdown resting zone chondrocyte model.

Besides inducing apoptosis, NO production also regulates the maturation of the growth plate chondrocytes (Teixeira et al., 2005).   The inhibitory effect of TP508 on NO production indicates that TP508 may delay the maturation of growth plate chondrocytes.   This prediction is confirmed by the fact that TP508 transiently blocks the differentiation of resting zone chondrocytes to growth zone chondrocytes (Schwartz et al., 2005).

Due to their same origin in embryonic development, growth plate is also used as a model for articular chondrocytes.   We have elucidated the sexual dimorphism of human articular chondrocytes in their response to 17β-estradiol (Kinney et al., 2005), based on a similar research in the rat growth plate model (McMillan et al., 2006).   In our current study, we have established that TP508 blocks NO production in chondrocytes.   Since NO production is related to the pathological degeneration of

articular chondrocytes in osteoarthritis (van den Berg, 2001), TP508 may also serve as

a potential drug against osteoarthritis.

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