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研究室で撮影した本人のスナップ写真、及び発表論文のコピーを添付

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研究テーマ CTLL-2細胞における、新しい増殖抑制剤SMDによる
アポトーシスの誘導

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表　有 ・ (無)　(学会名・内容)

(2) 学会誌等に発表した論文　有 ・ (無)　(雑誌名・論文名)

3．今後の研究計画

SMにより増殖を抑制された細胞の重要な酵素群であるプロテインキナーゼに与える影響を調べる。

4．研究指導者の意見

王銀娜さんは大変熱心に実験を行い，漢方薬中の物質の化学誘導体の一種が細胞増殖抑制作用をもち，細胞のアポトーシスを誘発することを証明し，現在学術誌に投稿すべく論文を作製中です．この論文が発表されると，アポトーシス誘発物質の新しいものとして注目を集めると思います．さらに，アポトーシスの機序を明らかにするための努力をしており，来年3月には医学博士の学位を取得する予定です．医学博士取得後は，中国へ帰り，研究と続けたいとの希望をもっており，日中医学の協力に役立つ人材に育ってくれることと思います．

研究指導者氏名　大谷周造　印

5．研究報告

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CTLL-2(ラットTリンパ球系)細胞における、新しい増殖抑制剤 Saliva Miltiorrhiza Derivative (SMD) によるアポトーシスの誘導

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SMD は、抗癌作用のある saliva miltiorrhiza （漢方薬）の分子構造を単純化した合成誘導体であり、構造的にはポリフェノール合成誘導体に属する。サイトカイン Interleukin-2 (IL-2) により増殖が維持される CTLL-2 細胞は、SMD による増殖抑制作用と、誘導されるアポトーシスを研究するのにいいモデルとなります。 SMD は培養細胞の増殖を抑制し、この抑制効果は、DNA 断片化率で測定されるアポトーシスの誘導と相関する事がわかった。このアポトーシスの過程では、セリン、スレオニンのリン酸化よりもむしろ 91, 80, 55kDa の各蛋白質のチロシンリン酸化が阻害され、同時にチロシンキナーゼ活性も抑制され、その結果としてプロテインキナーゼC (PKC) 活性の抑制を伴うこと、 即ち PKC は PTK のシグナル伝達の下流で阻害されるが、プロテインキナーゼ A (PKA) にたいしては SMD は効果がないことを示した。 bcl-2 蛋白の翻訳、bcl-2mRNA の発現及び酵素 caspase-3 の活性化はダウンレギュレーションを受けることがわかった。 SMD は Bcl-2 の発現と caspase-3 の活性を増加させたが、PKC の活性化剤である TPA (12-O Tetradecanoylphorbol 13-acetate) は、少なくともある部分で、SMD で誘導されるアポトーシスを弱めることができた。以上の結果により、SMD は新種の細胞増殖抑制剤であり、アポトーシス死を誘導するチロシンキナーゼの阻害剤であることを明らかにした。

Induction of Apoptosis in CTLL-2 Cells by a Novel Antiproliferative Agent, Saliva Miltiorrhiza Derivative(SMD)

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Abstract

Saliva miltiorrhiza derivative (SMD) is a simplified synthetic analogue of the principle of active saliva miltiorrhiza, and structurally it belongs to a polyphenol synthetic analogue. The cytotoxic T cell line driven by IL2 (CTLL-2 cells) was used as a model to study the antiproliferative potential and the development of apoptosis induced by SMD. We found that SMD can suppress the cell proliferation and this inhibitory effect by SMD was associated with the induction of apoptosis detected by DNA fragmentation assay. This apoptosis process is accompanied by inhibition of tyrosine phosphorylation of about 91, 80, 55-kDa proteins, rather than that of protein serine/threonine phosphorylation which dependent on IL2, and suppression of protein tyrosine kinase activity and subsequently inhibition of PKC activity that is in downstream of PTK without affecting PKA activity, as well as down-regulation of bcl-2 anti-apoptotic protein and bcl-2 mRNA, and activation of caspase-3 activity. A relation of bax protein levels to apoptosis was not appreciated. TPA, a protein kinase C activator was able to attenuate the apoptosis induced by SMD at least in part though increasing of bcl-2 protein expression and inhibiting of caspase-3 activity. Taken together, we conclude that SMD is a novel potent antiproliferative agent and a tyrosine kinase inhibitor that induced apoptotic cell death in CTLL-2 cells.

Key words: apoptosis, DNA fragmentation, protein phosphorylation, protein tyrosine kinase, protein kinase C, protein kinase A, bcl-2, bax, CPP32/Caspase-3 protease.

The abbreviations used are: IL2, interleukin-2; FCS, fetal calf serum; TCGF, T cell growth factor supplement; CTLL-2 cells, cytotoxic T lymphocyte; PTK, protein tyrosine kinase; PKC, protein kinase C; PKA, cAMP-dependent protein kinase (protein kinase A); TPA, 12-O-Tetradecanoylphorbo l 13-acetate; SMD, Saliva Miltiorrhiza Derivative.

Introduction

Saliva miltiorrhiza (Danshen) is a traditional chinese medicine which has been widely use for the treatment of cardiovascular diseases and inflammation for a long time. Saliva miltiorrhiza derivative (SMD) is a simplified synthetic analogue of the principle of active saliva miltiorrhiza, structurally, it belongs to a polyphenol synthetic analogue. Some studies have indicated that SMD is capable of a antioxidant and an antitumor activity in vitro(1,2). However, the biochemical and pharmacological mechanism for the SMD inhibitory effect has not yet been elucidated.

Apoptosis is the process of cell death relying on both gene induction and protein activation. A variety of genes involved in the regulation of apoptosis have been identified(3). Cells undergoing apoptosis display several morphological and biochemical changes, including cellular collapse, nuclear chromatin condensation, the formation of vesicular apoptotic bodies and internucleosomal DNA fragmentation. The morphological alterations are accompanied by a variety of biochemical changes, although the molecular mechanisms of apoptosis are still large unknown. Many signals and external stimuli regulate the apoptosis activity by interaction with a number of proteins and gene products, such as transcription factors, Myc, Apaf-1, wild-type 53 protein and bax were able to induce apoptotic cell death. and others bcl-2, bcl-$_{XL}$, Ras, Raf prevented cells from undergoing apoptosis(5-

6). Numerous disparate agents, for example toxins, glucocorticoids, protein kinase inhibitor, irradiation, growth factor withdrawal and anticancer drug induce apoptosis in variety of cell types (4). Protein phosphorylation may have multiple roles in the control of apoptosis process (10) and activation of specific protein kinase can in some circumstances protect against apoptosis (7). Recently, much attention has focused on the role of proteases related to interleukin-1 β converting enzyme (ICE, renamed caspases)(8,9) and it may be positive regulators in apoptosis. Now many experiments have been underscored that apoptosis can be activated by many types of cancer chemotherapeutic agents (11,12) and new therapeutic strategies aim to counteract dysregulation of apoptosis by pharmacological intervention.

CTLL-2 cells are the cell lines that undergo apoptosis upon growth factor deprivation (13). We used this cell line to investigate the effects of SMD on the inhibition of cell proliferation as it is a good model to study apoptosis. Our purpose in this study was to ascertain the molecular basis in CTLL-2 cells by examining a potent antiproliferative effect and the development of apoptosis by SMD in respect to IL2 induced-cell proliferation, apoptosis phenomenon, protein phosphorylation, protein kinases, as well as the alterations of expression of bcl-2/bax, CPP32/Caspase-3 activity.

Methods and Materials

Chemicals. Saliva miltiorrhiza derivative (SMD) was synthesized by professor Lianning-Li (Institute of Materia Medica, Chinese Academy of Medical Sciences) and were kindly provided. Recombinant human IL2 were from Shionogi Chmical Industries, Ltd.. 12-O-Tetradecanoylphorbol

13-acetate (TPA) was purchased from Sigma. Mouse monoclonal anti-phosphotyrosine antibody (Clone PY20) was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-phosphoserine antibody and rabbit polyclonal anti-phosphothreonine antibody were from ZYMED Laboratories, Inc (South Francisco, CA). Rabbit polyclonal anti-bcl-2 antiboby (Ab-2) was obtained from Calbiochem (Cambridge, MA). Rabbit polyconal anti-bax (P-19) was purchased from Santa Cruz Biotechnology, Inc. cDNA probe for bcl-2 was kindly provided by Dr Jeffrey Sklar (Stanford University School of Medicine. Stanford, California). [γ-^{32}P] ATP (3000Ci/mmol) and [α-^{32}P] dCTP (3000Ci/mol) were from NEN (Boston) and Amersham (England), respectivitly. All the other chemicals were of the purest grade commercially available.

Cell Culture. The cytotoxic T cell line CTLL-2 was cultured in a growth medium- RPMI 1640, 10% fetal calf serum (FCS) and 5% rat T cell growth factor supplement (TCGF) which is from the culture of rat spleen cells stimulated by concanavalin A (4 μg/ml) for 48h. Cells in their exponential growth phase were harvested, washed with RPMI 1640 medium, and suspended at 3.3×10^5/ml in the growth medium without TCGF in a humidified atmosphere of 95% air and 5% CO_2 for 8-10 h for starved cells. Then, cells were added to IL-2 (100 U/ml) and SMD in the various concentrations for the following experiments. The viable cells were determined by the trypan blue dye exclusion (14). Three representative fields were examined and viability was expressed as the percentage of cells excluding trypan blue dye.

DNA fragmentation assay. The internucleosomal DNA degradation was assay as described

previously (15) with slight modification. Briefly, 1.2×10^6 cells were seeded in $35mm^2$ culture dishes and treated with different concentrations of SMD and IL2 (100 U/ml) for different times. Cells were harvested and washed with ice-cold phosphate bufferred saline (PBS). DNA was extracted by the lysis buffer (20 μl of 10 mM EDTA, 50 mM Tris-Hcl (PH 8.0) containing 0.5% (w/v) sodium lauryl sarkosinate and 0.5 mg/ml proteinase K) for 1 h at 50°C. After then, 10 μl of 10 mg/ml RNase A was added to the each sample, and incubated for further 1 h at 50°C. Reaction was stopped by the addition of 10 μl 10 mM EDTA (PH 8.0). Sample were heated at 70°C for 20 min. The total DNA from each sample was loaded in 1.2% (w/v) horizontal agarose gel containing 0.1 μg/ml ethidium bromide. The gel was run in TBE buffer (90 mM Tris, 90 mM borate and 2 mM EDTA) at 2.5 v/cm, and photographed under UV light.

Protein kinase Assay. Kinase activities of protein tyrosine kinase (PTK), protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) were assessed using a SignaTECT™ PTK, PKC and PKA Assay System Kit (Promega, U.S.A) using PTK Biotinylated Peptide Substrate (for PTK), Biotinylated Peptide Neurogranin (for PKC) and Biotinylated Peptide Kemptide (for PKA) as substrates, respectively, according to the manufacturer's instructions. For detecting the three of protein kinase activities in intact cells, the cells were incubated in RPMI 1640 medium treated by with or without IL2 and SMD for various periods of time (0 min to 120 min) and harvested by washing with PBS. The cells were lysed by the extract buffer to determine the protein kinase activities. For measuring the kinase activities in cell lysate, the cells treated with or without IL2 were incubated in 10 min and lysed by the extraction buffer, and centrifugated and save the supernatant. 5 μl of cell lysate

was added to the Reaction Mixture containing DSMO solution of SMD (1 μl) at various concentrations in a final volume of 25 μl for determining the protein kinase activities, respectively. The biotinylated, ^{32}P-labeled peptides were recovered from the Reaction Mixture with the SAM^{2TM} Biotin Capture Membrane and phosphorylation of the peptides were determined by a scintillation counting (LS 6500, Beckman). Protein concentration was determined by a Bio-Rad protein assay kit (Bio-Rad, Richmond, VA) using bovine serum albumin as a standard.

Western Blot Analysis. 2.5×10^6 cells were harvested and washed with ice-cold PBS, and solubilized in a lysis buffer (1% Triton X-100, 50 mM Tris (PH7.4), 1 mM $NaVO_4$, 0.5 mM EGTA, 200 mM NaCl, 60 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1.5 μg/ml aprotinin) by vortexing and incubating on ice for 10 min. The lysates were centrifuged at 1,500g for 10 min, and supernatants were further clarified by centrifugation at 100,000g for 30 min. The supernatants were saved and the protein contents were determined with the Bio-Rad protein assay using bovine serum albumin as a standard. Twenty-forty micrograms of protein per lane respectively, was electrophoresed on 8.75-13.8% polyacrylamind-SDS minigels (16) in SDS sample buffer. The proteins were then transferred to polyvinylidene difluoride sheet (PVDF membrane; Pall Biosupport Division, Port Washington, NY) by electroblotting at 18V for 1 h. The membrane was blocked for 2-3 h in a blocking buffer (3% BSA and 0.25% normal horse serum in TBS). The blots were detected by anti-phosphotyrosine (1:1000), anti-phosphoserine (1:500), anti-phosphothreonine (1:500), bcl-2 (1:20) and bax (1:200) antibodies. Blotted membrane was incubated with a peroxidase-conjugated avidin (ABC kit; Vector Laboratories, Burlinggame, CA) and developed in AEC solution (0.8%

amino-ethylcarbazole and 0.1M acetate, PH 5.2). The bands that recognized by each antibodies were scanned with a laser densitometer.

Northern Blot Analysis. 5×10^6 cells were harvested after treatment of IL2 and SMD, and total RNA was extracted by the method of the acid guanidinium thiocyanate-phenol-chloroform method (17). RNA (20 μg/lane) was electrophoresed through a 1% agarose gel containing 6% formaldehyde and blotted onto a nylon membrane (Hybond-N+ membrane, Amersham), The membrane was prehybridization at 42°C for 2 h in a solution containing 50% formamide, 3× standard saline citrate (SSC), 0.1% SDS, 20 mg/ml boiled salmon sperm DNA, and 1× Denharht's solution. Hybridization was performed for 16 h at 42°C in the same prehybridization solution with bcl-2 (18) and 18s-rDNA probe(19). The probes were labeled with [α-^{32}P] dCTP using a random DNA labelling kit (Amersham LIFE SCIENCE, England). After hybridization, the membrane was washed three times at 42°C for 20 min in 2×SSPE and 0.1% SDS washing buffer, once for 30 min in 0.1 × SSPE and 0.1% SDS washing buffer at room temperature. Then the membrane was subjected to autoradiography and the density of each band was assessed semiquantitatively using a laser densitometer. The densities of bcl-2 mRNA bands were normalized relative to the value of the 18s-rDNA.

Assay of CPP32 activity. CPP32 activity was measured using a CPP32/ Caspase-3 Colorimetric Protease Activity Assay Kit (MBL, Nagoya, Japan) according to the recommendations of manufacturer (20). Briefly, 2×10^6 SMD-treated CTLL2 cells at the indicated time were harvested

and washed in PBS, suspended in 50 μl Cell Lysis Buffer and incubated on ice for 10 min. The lysates were centrifugated at 10,000g for 1 min. The equal amounts of protein contents in each samples were incubated with substrate DEVD-pNA (chromophore p-nitroanilide, 200μM) at 37°C for 1 h. The pNA light emission was monitored using a spectrophotometer (DU640, Beckman) at 400 nm. Date were expressed as absorbance of 400 nm wavelength.

Statistical analysis. The date were expressed as the means $\pm$ SD (Standard deviation). The significance of the experiment date were validated using the Studen's-t test. The differences were considered as to be significant when $p<0.05$.

Results

1. Antiproliferative effects of SMD

Cell viability was investigated to ascertain whether or not SMD can affect the cell proliferation in CTLL-2 cells. About 50% of cells died by 16 h after deprivation of IL2 and cell viability is still above 91% by 16 h after the addition of IL2, SMD (50 μg/ml) plus IL2 caused a markedly reduction of cell viability in a time dependent manner, and almost 50% and 70% of cells died by 12 h and 16 h, respectively, as measured by trypan blue dye exclusion (Fig 1A). The cell viability was inhibited in a does-dependent manner (Fig 1B) when IL2 withdrawal or the addition of SMD plus IL2. The cell viability was significantly inhibited at 20 μg/ml SMD ($p<0.05$).

2.Effects of SMD on DNA fragmentation

The hallmark feature of programmed cell death is the formation of DNA fragments. Thus, the

analysis of DNA fragmentation by agarose gel electrophoresis was observed to understand whether or not SMD cytotoxic effect was able to induce apoptosis in CTLL-2 cells. Fig 2A shows that DNA fragmentation was visible after 12 h of IL2 deprivation, and after 16 h of IL2 deprivation, there was a marked intensification in the oligonucleosomal ladder. However, after 16 h of the cells treated by IL2, no DNA ladder was observed. When cells were treated with herbimycin A or SMD plus IL2 for 12 h , DNA degradation was induced. The date indicated that SMD induced apoptotic cell death is in a time-dependent manner. Fig 2B shows that the induction of apoptosis by SMD is in a dose-dependent manner. At 20 μg/ml SMD plus IL2, DNA ladder was appeared and at 50 μg/ml SMD plus IL2, the intensity of DNA ladder was visible. Otherwise, in the presence of IL2 and 0.1% DMSO (a vehicle), no DNA fragmentation was detectable. Taken together, These date suggest that SMD was able to abolished the IL-2-induced protection against cell death and induced apoptosis in CTLL2 cells.

3. Effects of SMD on protein phosphorylation induced by IL2

The phosphorylated-proteins were investigated to understand the relationship of protein phosphorylation and apoptosis induced by IL2 deprivation or SMD in CTLL-2 cells. We firstly examined the role of phospho-tyrosine containing proteins. As seen in Fig 3A and Fig 4A,B,C, the tyrosine phosphorylation of 91, 80 and 55kDa proteins were dependent on IL2, and SMD was able to inhibit tyrosine phosphorylation of that proteins. Next, we detected the presence of serine and threonine phosphorylated-proteins to understand whether or not the serine/threonine phosphorylation involved in the cell proliferation induced by IL2 and SMD can suppress these serine/threonine

phosphorylated-proteins. As shown in Fig 3B,C and Fig 4D,E, the serine phosphorylation of about 46.5kDa protein and threonine-phosphorylation of about 31.5kDa protein were increased after stimulation of IL2, and these serine/ threonine phosphorylated-proteins induced by IL2 were not inhibited by SMD. These date suggested that SMD inhibited the tyrosine phosphorylation of proteins, however does not affect serine and threonine phosphorylation of proteins which were dependent on IL2.

4. Effects of SMD on protein kinase activities

Fig 5A is the time-course of protein tyrosine kinase activity (PTK) in the intact cells, and shows that an initial increase of PTK activity was observed at 5 min and maintaining higher levels by 120 min in the cells treated with or without IL2. However, in the cells with SMD (50 μg/ml) plus IL2, PTK activity was underwent lower activity compared with in the cells of minus or plus IL2. Fig 5B is the does-response of PTK activity in the cell lysate by direct incubation of SMD at various concentrations and shows that SMD inhibited PTK activity in a dose-dependent manner both in the cell lysate with or without IL2. SMD substantially inhibited PTK activity at the concentration of 10 mg/ml ($p<0.01$). Fig 6A shows that protein kinase C activity (PKC) by phospholipid activation was reduced in the intact cells treated with SMD (50 μg/ml) plus IL2 compared to in the cells treated with IL2 ($p<0.05$). There were no significant difference on PKC activity both in the intact cells treated with or without IL2. On the contrary, PKC activity did not decrease by direct incubation with SMD in the cell lysate ($p>0.05$, shown in Fig 6B). Fig 7A and B shows that cAMP-dependent protein kinase activity (PKA) in the presence of cAMP was not affected both in the intact cells and

cell lysate treated by SMD. These results indicated that SMD was able to inhibit PTK activity both of dependent or independent-IL2. PKC activity was not inhibited directly, but inhibited indirectly by SMD. No inhibition of PKA activity was observed by SMD in CTLL-2 cells, and suggested that SMD is a protein tyrosine kinase inhibitor.

5. Effects of SMD on the expression of Bcl-2 homologous proteins and Bcl-2 mRNA

To explore the possible role of bcl-2 and bax in the SMD induced-apoptosis, we examined the effects of SMD on bcl-2 and bax protein expression analyzed by western blot analysis. Fig 8A,B shows that there was a significantly increase in bcl-2 protein at 4 h in the cells trested with IL2, and it continued to reach a highest level up to 12 h, and then bcl-2 protein subsequently underwent decrease. Bcl-2 protein was suppressed when SMD was added in the cultures. Fig 9A,B shows the detectable levels of bcl-2 protein began to be decreased by SMD (10-20 μg/ml) treatment, and the inhibitory effect of SMD on bcl-2 protein was in a dose -dependent manner. As indicated in Fig 10, the incubation of CTLL-2 cells without IL2 which was similar to SMD-induced apoptosis or with IL2 failed to induced a change in bax expression, indicating that there is no alteration in bax protein expression on the apoptosis process in CTLL-2 cells. We further examined the level of bcl-2 mRNA by northern blot analysis in order to determine whether the suppression of bcl-2 protein is due to a decrease in bcl-2 molecules. As shown in Figure 11, there was a significantly decrease in bcl-2 mRNA expression in the cell incubation either without IL2 (lane1) or with SMD plus IL2 (lane3) in comparison to IL2 alone (lane2).

6. Effect of TPA on apoptosis induced by SMD

TPA (phorbol ester, 12-O-Tetradecanoylphorbol 13-acetate) is a protein kinase C (PKC) activator, as well as considered as a promoter carcinogen. Some studies demonstrate that PKC activator inhibit apoptosis (21-23). So that we investigated whether or not TPA is able to block DNA fragmentation induced by SMD. As shown in Fig 12, the cells treated with 20 μg/ml SMD showed some DNA fragmentation appeared (lane3), and TPA inhibited this DNA fragmentation (lane4). In contrast, TPA did not block DNA fragmentation induced by 50 μg/ml SMD (lane6). The date indicated that TPA have an inhibitory effect on the apoptosis induced by SMD at dose of 20μg/ml. Next, we examined the expression of anti-apoptotic bcl-2 protein and caspase-3 activity to further characterize some mechanism in TPA blocked-apoptosis. As shown in Fig 13A,B, western blot analysis revealed that TPA could restore the inhibition of bcl-2 protein expression caused by 20 μg/ml SMD (lane 4). Fig 14 shows that the caspase-3 activity was increased after the withdrawal of IL2 and treatment of SMD either 20 μg/ml or 50 μg/ml($p<0.01$) in comparison with IL2 treatment alone. TPA restored the increase in caspase-3 activity caused by 20 μg/ml SMD ($p<0.05$), however, did not reverse the elevation of caspase-3 activity treated by 50 μg/ml SMD ($p>0.05$). These results indicated that caspase-3 activity is involved in apoptosis process and TPA was able to attenuate apoptotis cell death at least in part through increasing of expression of bcl-2 protein and inhibiting of caspase-3 activity in CTLL-2 cells.

Discussion

Apoptosis can be induced by diverse signaling pathway in a variety of cell lineages. In this report, we investigated the mechanism of antiproliferative effect of SMD (a new polyphenol synthetic analogue) and found that SMD can suppress tyrosine kinase activity and the cell proliferation. This inhibitory effects by SMD were associated with the induction of apoptosis. DNA fragmentation is widely used to assess apoptosis. The present study shows that DNA fragmentation was observed in time and dose-dependent manner, and required at least 12 h and 20 μg/ml of exposure to SMD after the addition of IL2 (Fig 2). This is consistent with the time and dose-fashion of inhibition of cell viability as assessed by trypan blue dye exclusion (Fig 1).

The protein phosphorylation is by far the most common mechanism for posttranslational regulation of protein function, therefore, the role of protein phosphorylation during apoptosis have been described in many reports. Ohoka and coworks have demonstrated, using okadaic acid, that protein dephosphorylation is an essential step for glucocorticoid-induced apoptosis in murine T-cell hybridomas (24). A correlation has been observed between the dephosphorylations of protein by downregulation of cPKC and the induction of apoptosis in prolong culture of promonocyte leukaemia cells (U937 cells) (25). During Fas-mediated apoptosis, tyrosine phosphorylation at about 56-60, 70 and 100-110 kDa was increased in a human T-cell line (26). Barkett et al. have documented that cleavage of I κ B-alpha by CPP32 was blocked by serine phosphorylation of I κ B-alpha (27). Exposure of U936 cell, a human myeloid leukemia cell line to etoposide stimulated the tyrosine-phosphorylation of 30 and 60 kDa proteins in etoposide-induced apoptosis (28). It is well known that IL2 receptor (IL-2R) have intracellular domain to which associates an intrinsic protein-tyrosine

kinase (PTK) (29). Turner et al. reported that the activity of serine/threonine specific Raf-1 kinase can be regulated by tyrosine phosphorylation in CTLL-2 cells (30). Thus, tyrosine phosphorylation is considered often occur early in signalling cascades in the many survival factor receptors, and then, their effect can be strongly amplified via, e.g., serine/threonine kinases (10). In our study, we have demonstrated that of these phosphorylated-proteins, the about 91, 80 and 55kDa tyrosine phosphorylated-proteins, 46.5kDa serine phosphorylated-protein and 31.5kDa threonine phosphorylated-protein were dependent on IL2 in CTLL-2 cells, and implied that the cell proliferation in IL2-driven CTLL-2 cells is involved in both tyrosine phosphorylation and serine/threonine phosphorylation pathways. All of tyrosine phosphorylation of about 91, 80 and 55kDa proteins which were dependent on IL2 were suppressed in SMD-induced apoptosis process, althrogh the nature of these proteins are as yet unidentified. Our findings suggested that SMD induced apoptosis through the inhibition of tyrosine phosphorylation rather than that of serine/threonine phosphorylation, and tyrosine phosphorylation play an important role on the apoptotic process in SMD-induced apoptosis.

We next examined the effects of SMD on protein phosphorylating enzymes- protein kinase activities in order to explore the possible role of protein kinases, because SMD could affect the tyrosine phosphorylation proteins on apoptosis process induced by SMD in CTLL-2 cells. Many studies have been shown that tyrosine kinase activity can trigger the cell proliferation and apoptosis process in multiple cells. The PTK inhibitors, herbimycin and genistein prevented the radiation-induced phosphorylation and inhibited apoptosis, and suggesting that protein tyrosine kinases play a

pivotal role in the apoptosis induced by radiation (31). Bae et al, recently reported that the protein tyrosine kinase activation is required for upregulation of Fas ligand (FasL) expression and subsequent mediation of Fas death signaling account for aburatubolactam C (a novel inhibitor of supperoxide anionic generation) induced apoptosis in Jurkat cells (32). In the present study, three of protein kinase activities were investigated, protein tyrosine kinase activity (PTK) which are responsible for less than 1% of protein phosphorylation within cells; and two serine/threonine kinases, protein kinase C (PKC) and cAMP-dependent protein kinase(PKA) activities. We found that SMD significant inhibited PTK activity by the concentration of 10μg/ml. On the other hand, SMD did not affected PKA activity and PKC activity in the cell lysate, although when exposing intact cells to SMD, PKC activity was inhibited. Thus, suggesting that SMD may act through inhibition of protein tyrosine kinase activity, and subsequently suppressed PKC activity that is in downstream of PTK to induce cell apoptotic death. Our finding also indicated that SMD is a novel protein tyrosine kinase inhibitor.

The bcl-2 family including its homologues, and caspase family of cysteine proteases play important roles in regulating apoptotic cell death induced by a variety of stimuli in mammalia cells (33-36). Bcl-2 gene family, acting at a central checkpoint in the apoptotic pathway, can suppress apoptosis. while, caspase family of cysteine protease facilitate apoptosis that functional site is in the downstream of bcl-2 influence (37). In this study, we examined the effect of SMD-induced apoptosis on the expression of bcl-2 and bax. We found that a obvious amounts of bcl-2 protein (Fig 8,9) and mRNA (Fig 11) were seen after 12 h of treatment with IL2. However, both bcl-2 protein and mRNA were inhibited

by SMD treatment. In contrast, bax protein was not changed when the cells exposure to IL2 deprivation or treatment of IL2 or SMD, suggesting that SMD induced apoptosis through the alteration of bcl-2 protein level and mRNA expression, but not through that of bax protein level. The date presented herein argues the ratio of the expression of bax to bcl-2 play a predominant role in the apoptosis process, up-regulation of bax or down-regulation of bcl-2 facilitates apoptosis (38). Recently, in some system, bax binding bcl-2 was not sufficient to prevent apoptosis and the overexpression of bcl-2 or bcl-XL can repress apoptosis in the absence of bax (39,40). These observation are concomitant with our findings. The mechanism of bcl-2 on anti-apoptotic properties has also been elusive. Bcl-2 is considered as a phosphoprotein. It may be regulated by phosphorylation (41,42). Bcl-2 prevent apoptosis also through blocking cytochrome C release from mitochondria (43). We next measured caspase-3/CPP32 activity to understand whether or not caspase family of cysteine proteins contribute to the apoptosis process induced by SMD in CTLL-2 cells, because overexpression of these proteases frequently results in apoptosis (44) and bcl-2 and caspase inhibitor can abrogate the activation of caspase leading to cell survival(37). Rescently, Hughes et al. reported that disruption of the natural K^+ electrochemical gradient suppressed the activity of both caspase and apoptosis induced by glucocorticoid dexamethasone, thapsigargin and staurosporin (36). In present study, we shows that an increase in caspase-3 activity in response to both IL2 deprivation and SMD-treatment was observed (Fig 14). These results suggested the caspase-3 protease is involved in the apoptosis process in CTLL-2 cells, although the involvement of ICE family proteases other than caspase-3 on the apoptosis process is not yet clear.

Protein kinase C (PKC) is intimately involved in the regulation of cell proliferation and cell survival (45). TPA, a protein kinase C activator, have been reported to protect several cell lines from apoptosis induced by a variety of agents (46,47). Our studies shows that PKC activity was inhibited through suppressing PTK activity in apoptotic cell death by SMD and TPA can attenuate apoptotic cell death induced by SMD. This was interpreted to be due to PKC activation. Now it is unclear that why activation of PKC blocked apoptosis induced by different agents. A correlation has been observed between the dephosphorylation and downregulation of cPKC and the induction of apoptosis, and suggesting PKC provides a survival signal that can be supplied by PKC α alone (25). Knox et al (48) reported that the PKC isozyme distribution are associated with the regulation of apoptosis in stratified squamous epithelium and coincident with the presence of bcl-2 protein. We also shown that TPA reduced the extent of cell death at least in part by increasing the expression of the bcl-2 anti-apoptotic protein and inhibiting of caspase-3 activity. Our observations are concomitant with above reports.

In summary, in this study, we conclude that SMD possesses potential antiproliferative activity that rapidly induces apoptotic cell death in CTLL-2 cells. This apoptosis process is correlated with the inhibition of protein tyrosine phosphorylation, protein tyrosine kinase and subsequently suppression of protein kinaes C that is in downstream of PTK without affecting PKA activity, as well as the decrease in bcl-2 protein and increase in caspase-3 activity, and suggesing that SMD is a protein tyrosine kinase inhibitor and expected to be a promising candidate for novel antiproliferative therapeutics. Our results also imply that multiple parallel and possible independent apoptotic pathway

exist in CTLL-2 cells rather than a single linear pathway.

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Fig 1. Effect of SMD on the cell viability in CTLL-2 cells. The cells in the exponential growth were harvested and starved in the fresh RPMI 1640 with 10% FCS without TCGF for 8-10 h. SMD were added to the culture for 30 min before the addition of IL2. Viable cells were measured by trypan blue exclusion. Date are mean ± SD of three determinations. (A) Kinetics. † $p<0.05$ and †† $p<0.01$ compared to time 0 h. * $p<0.05$ and ** $p<0.01$ compared to IL2 alone at each time point. (B) Dose-response. * $p<0.05$ and **$p<0.01$ compared to IL2 alone.

Fig 2. Effect of SMD on DNA fragmentation in CTLL-2 cells. The cells were incubated and harvested after the addition of SMD and IL2 for agarose gel electrophoresis analysis as described in Materials and Methods. (A) kinetics. SMD is 50 μg/ml, and Herbimycin A (Hb A) is 0.25 μM/ml. (B) Dose-response. The cells were cultured up to 16 h. Lane 1: without IL2, Lane 2: with IL2, Lane 3: Herbimycin A (0.25μM/ml) plus IL2, Lane 4: 0.1% DMSO plus IL2, Lane 5: SMD 0.5 μg/ml plus IL2, Lane 6: SMD 5 μg/ml plus IL2, Lane 7: SMD 10 μg/ml plus IL2, Lane 8: SMD 20 μg/ml plus IL2, Lane 9: SMD 50 μg/ml plus IL2, Lane 10: SMD 100 μg/ml plus IL2.

Fig 3. Effects of IL2 and SMD on tyrosine, serine and threonine phosphorylated-proteins in CTLL-2 cells. The cells were incubated stimulated with IL2 and SMD and harvested at 10 min. Cellular proteins were extracted and separated by SDS-PAGE electrophoresis. The tyrosine, serine and threonine phosphorylated-proteins were detected by western blot analysis with anti-phosphotyrosine, anti-serine and anti-threonine antibodies as described in Materials and Methods. M: Molecular size of protein was measured using Protein Molecular Weight Marker as the standard. The results are from three independent experiments. (A) Protein tyrosine phosphrylation. (B) Protein serine phosphorylation. (C) Protein threonine phosphorylation.

Fig 4. The extent of tyrosine, serine and threonine phosphorylated-protein was measured by a laser densitometer. (A) Phospho-tyrosine 91 kDa protein. (B) Phospho-tyrosine 80 kDa protein. (C) Phospho-tyrosine 55 kDa protein. (D) Phospho-serine 46.5 kDa protein. (E) Phospho-threonine 31.5kDa protein.

Fig 5. Effect of SMD on protein tyrosine kinase activity (PTK) in CTLL-2 cells. (A) Effect of SMD on PTK activity in intact cells. SMD (50 μg/ml) and IL2 were added to the cell culture at 37°C in the indicated time to measure PTK activity as described in Materials and Methods. *p<0.05, **p<0.01 and °p>0.05 compared to IL2 values at each time point. ‡p<0.05 and † p>0.05 compared to time 0 hr. (B) Effect of SMD on PTK activity in the cell lysate. NS not significant difference and ** p<0.01 compared to with or without IL2 values, respectively.

Fig 6. Effect of SMD on protein kinase C activity (PKC) in CTLL-2 cells. (A) Effect of SMD on PKC activity in intact cells. SMD (50 μg/ml) and IL2 were added to the cell culture for 10 min at 37°C and harvested to detect PKC activity as described in Materials and Methods. **p<0.05 and NS p>0.05 compared to IL2 values. (B) Effect of SMD on PKC activity in the cell lysate. † not significantly different compared to without IL2 values.

Fig 7. Effect of SMD on cAMP-Dependent Protein Kinase Activity (PKA) in CTLL-2 cells. (A) Effect of SMD on PKA activity in intact cells. SMD (50 μg/ml) and IL2 were added to the cell culture for 10 min at 37°C and harvested to detect PKA activity as described in Materials and Methods. There was no significant difference in each groups (p>0.05). (B) Effect of SMD on PKA activity in the cell lysate. † not significantly different compared to without IL2 values.

Fig 8. Time-course of SMD effect on the expression of bcl-2 protein in CTLL-2 cells. SMD (50 μg/ml) and IL2 were added to the cultures, and the cells were incubated and harvested at indicated times to detect the level of bcl-2 protein. (A) Western blot analysis. (B) Densitometric analysis of bcl-2 expression using a laser densitometer.

Fig 9. Dose-response of SMD effect on the expression of bcl-2 protein in CTLL-2 cells. SMD at indicated concentrations and IL2 were added to the cultures. The cells were incubated and harvested at 12 h for the detection of bcl-2 protein. (A) Western blot analysis. (B) Densitometric analysis of bcl-2 expression using a laser densitometer.

Fig 10. Effect of SMD on the expression of bax protein in CTLL-2 cells. SMD and IL2 were added to the cultures, and the cells were incubated and harvested at 12 h for the detection of bax protein. (A) Western blot analysis. (B) Densitometric analysis of bax expression using a laser densitometer.

Fig 11. Effect of SMD on the expression of bcl-2 mRNA in CTLL-2 cells. The total RNA was isolated from the cells treated by SMD and IL2 at 12 h. mRNA was analyzed by Northern blot analysis. (A) Northern blot hybridization of bcl-2 mRNA. Lane 1: without IL2, Lane 2: with IL2, Lane 3: SMD 50 μg/ml plus IL2. (B) The relative amounts of bcl-2 mRNA expression, as determined by a laser densitometer, and standardized to the amount of 18s-cDNA mRNA and expressed in arbitrary units.

Fig 12. Effect of TPA on DNA fragmentation in the apoptosis induced by SMD. SMD was added to the cultures for 30 min before the addition of IL2 and TPA. The cells were incubated and harvested at 16 h. DNA fragmentation was analyzed as described in Materials and

Methods. Lane 1: without IL2, Lane 2: with IL2, Lane 3: SMD (20 μg/ml) plus IL2, Lane 4: SMD (20 μg/ml) plus IL2 and TPA (0.05 μM /ml), Lane 5: SMD (50 μg/ml) plus IL2. Lane 6: SMD (50 μg/ml) plus IL2 and TPA (0.05 μM/ml).

Fig 13. Effect of TPA on the expression of bcl-2 protein in the apoptosis induced by SMD. SMD (20 μg/ml), IL2 and TPA were added to the cultures. The cells were incubated and harvested at 12 h for the detection of bcl-2 protein. (A) Western blot analysis. (B) Densitometric analysis of bcl-2 expression using a laser densitometer.

Fig 14. Effect of SMD and TPA on caspase-3 activity in CTLL-2 cells. SMD, IL2 and TPA were added to the cultures. The cells were incubated and harvested at 16 h to measure caspase-3 activity as described in Materials and Methods. *$p<0.01$ compared to IL2 alone. †$p<0.05$ compared to SMD 20 μg/ml plus IL2. ††$p>0.05$ compared to SMD 50 μg/ml plus IL2 .

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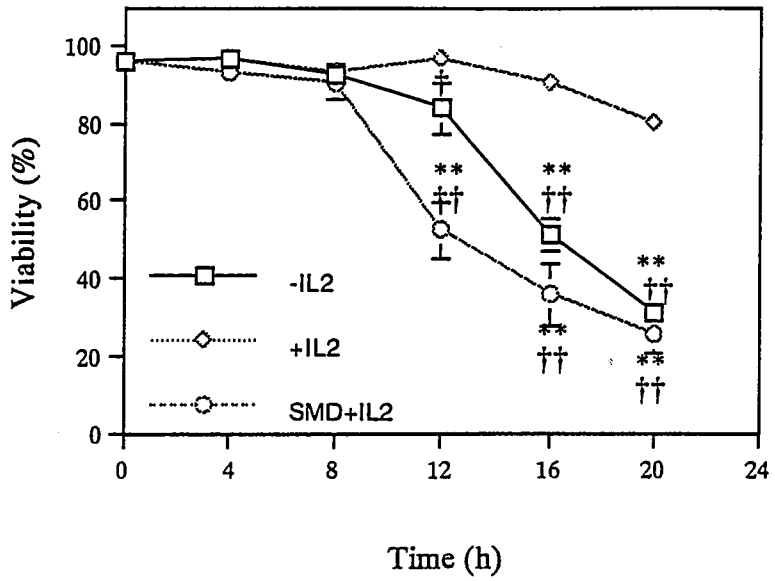
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SMD+IL2
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††
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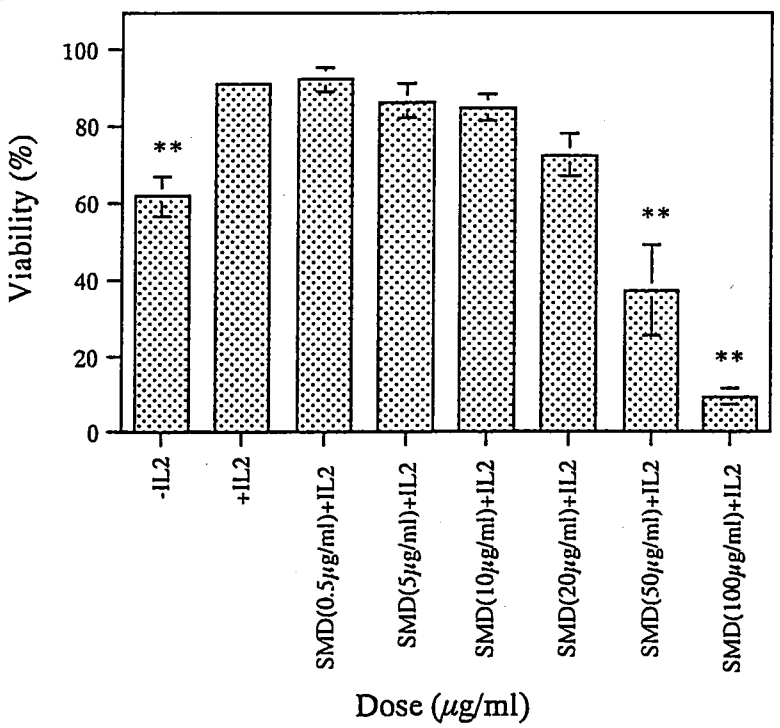
Time (h)

B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Dose (μg/ml)

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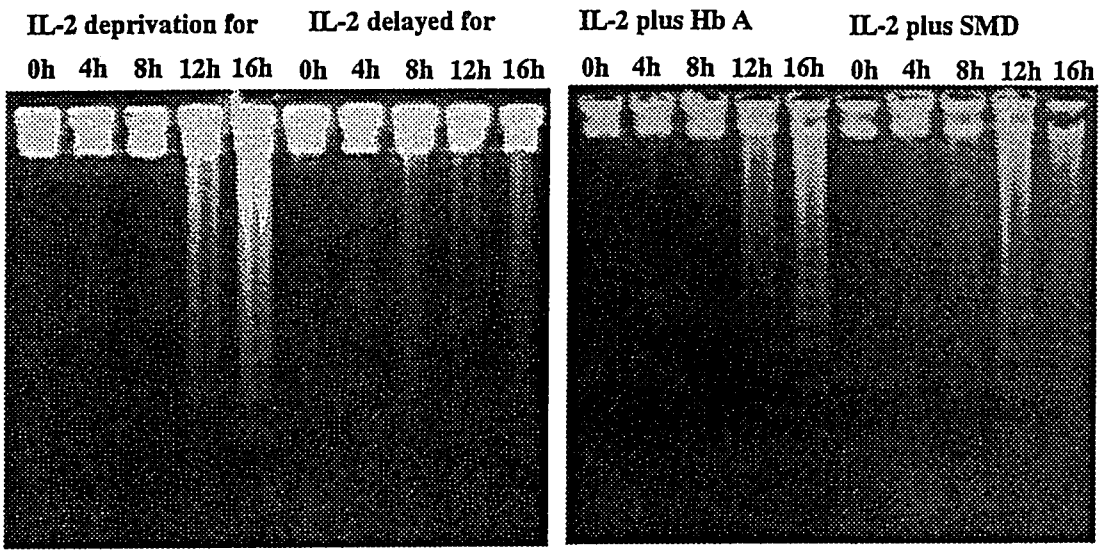
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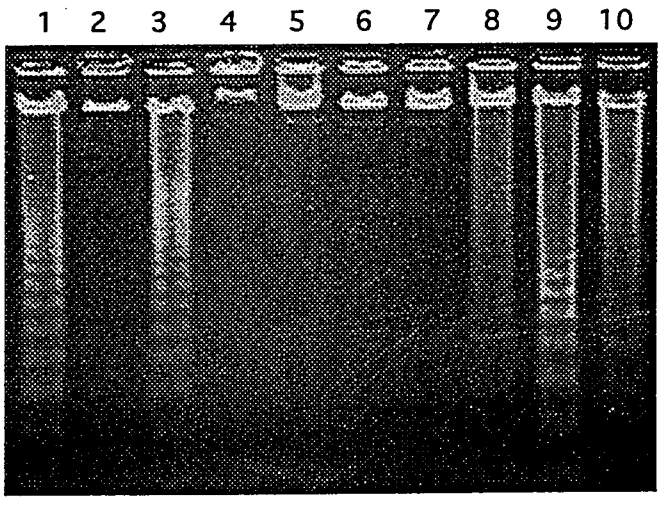
B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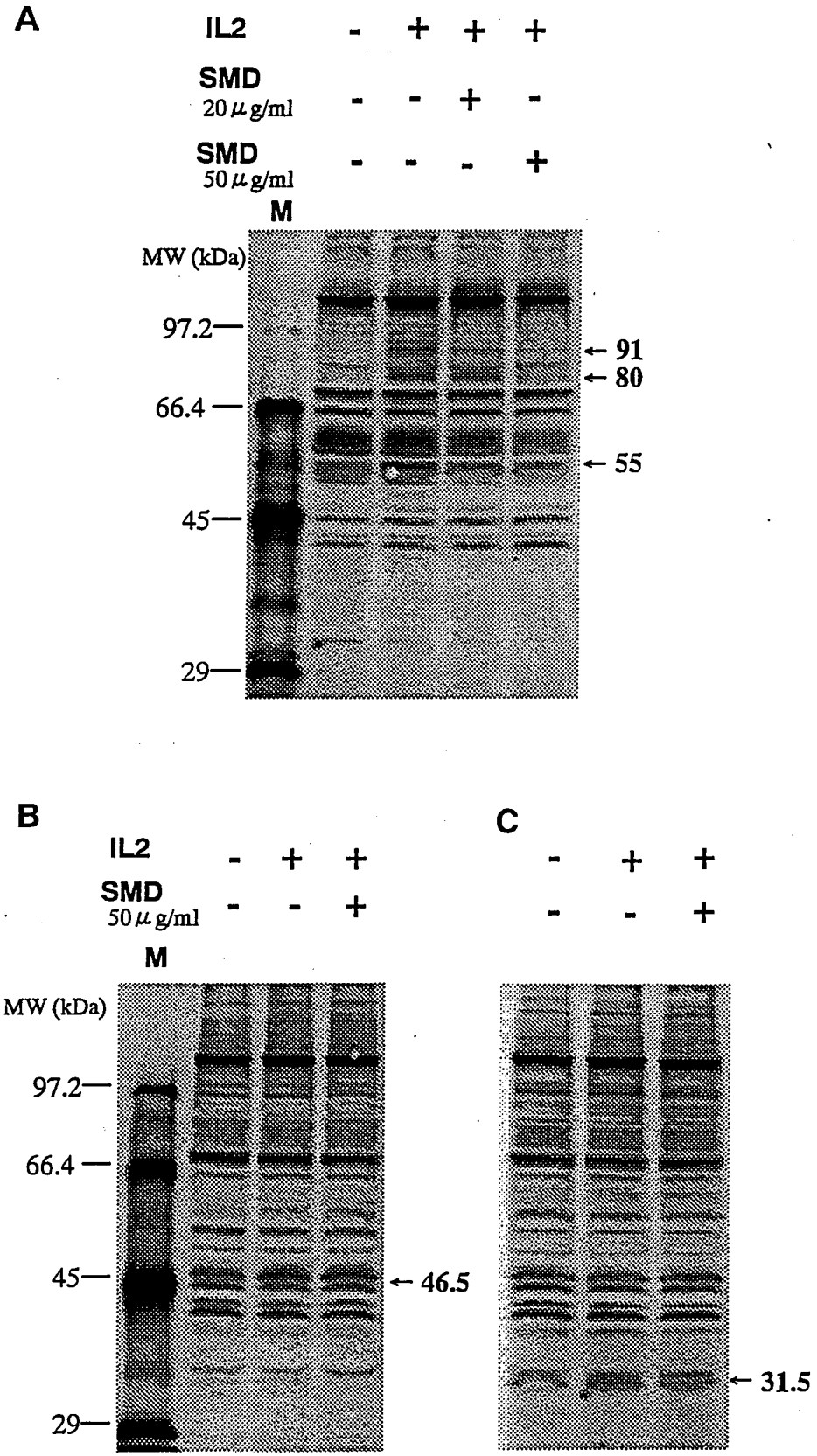
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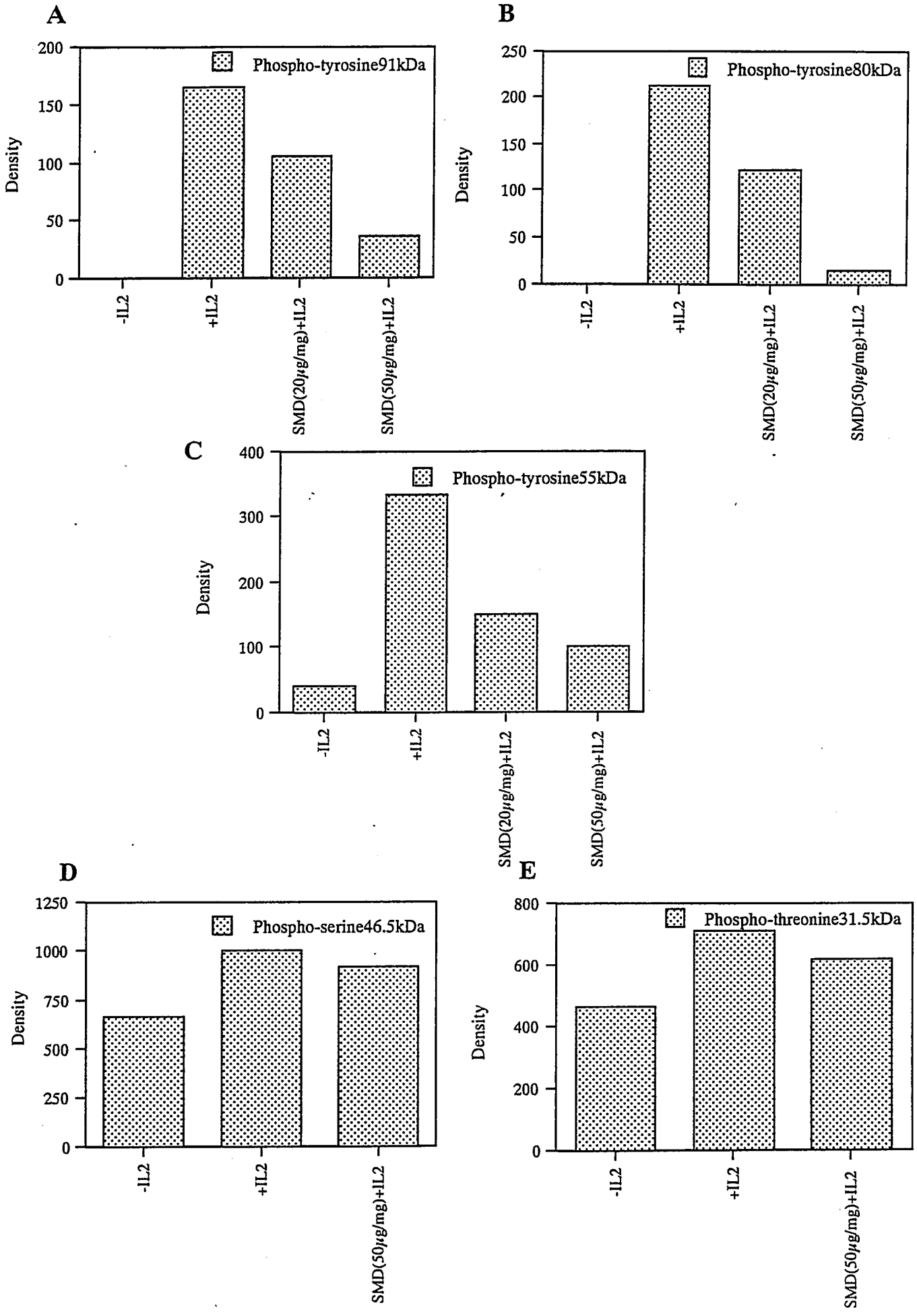
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150
100
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+IL2
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SMD(50μg/mg)+IL2
C
Phospho-tyrosine55kDa
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200
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D
Phospho-serine46.5kDa
Density
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750
500
250
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SMD(50μg/mg)+IL2
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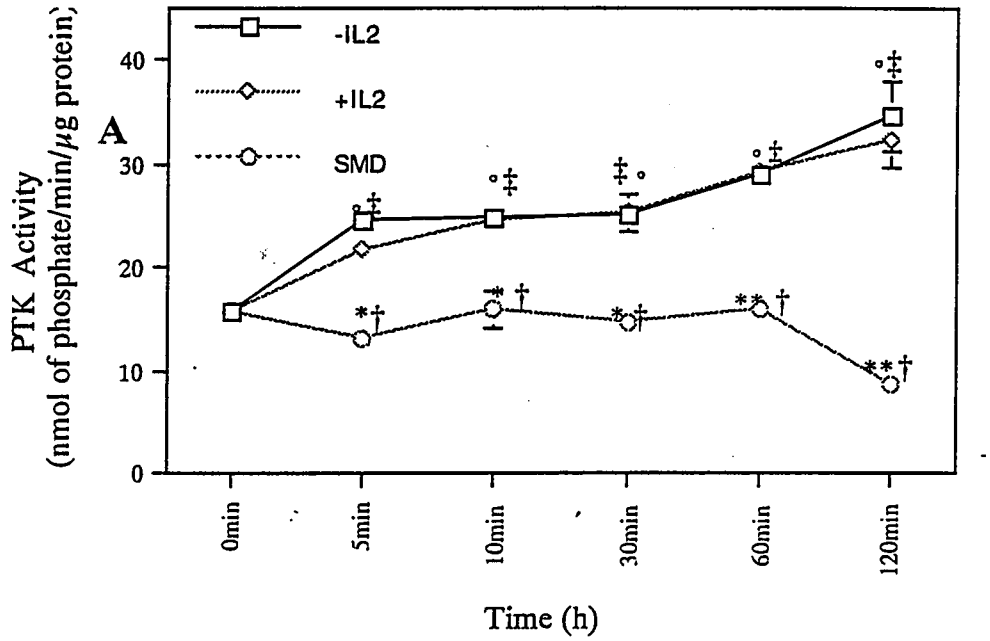
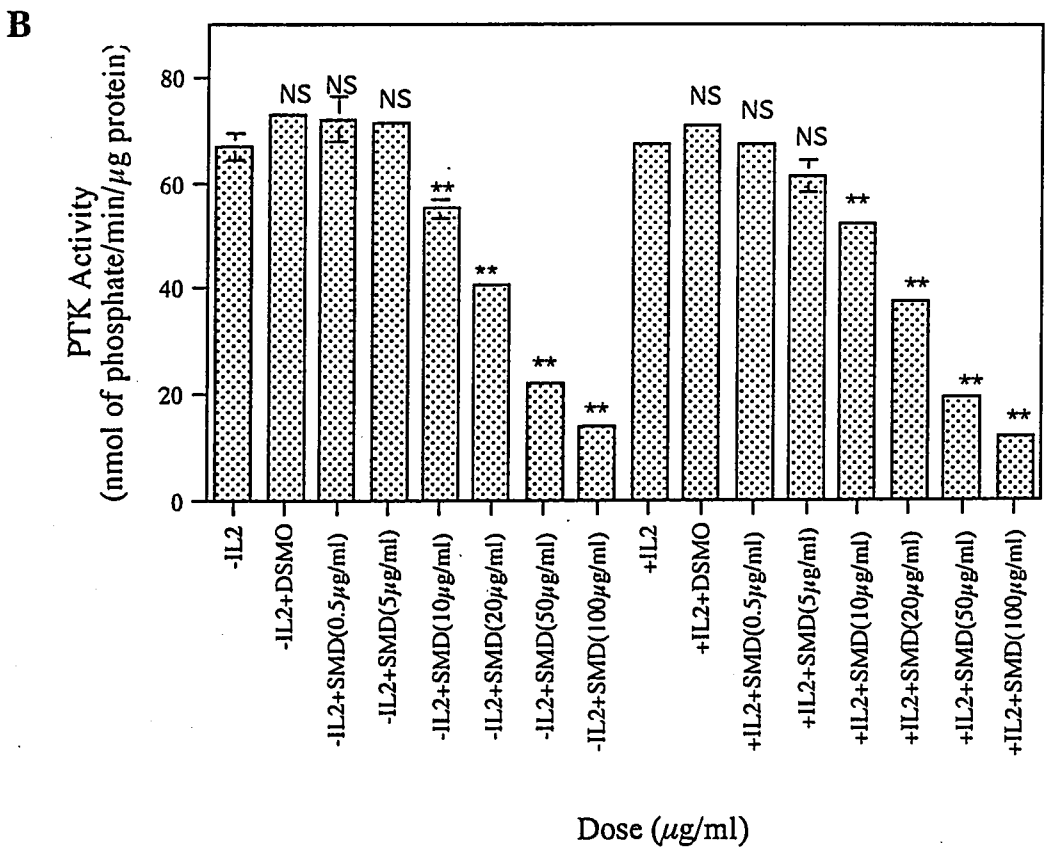
Intact Cell



Cell Lysate



Intact Cell

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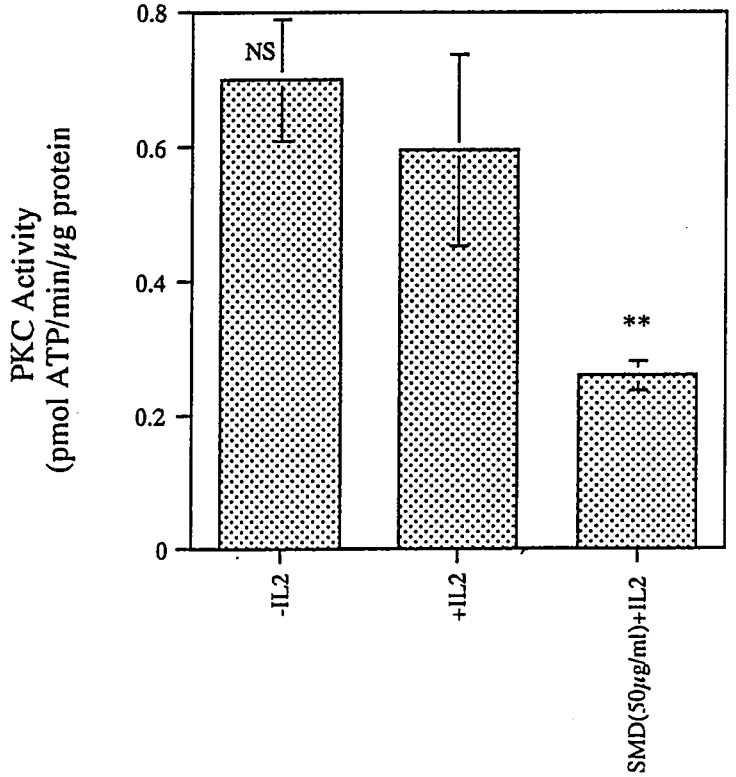
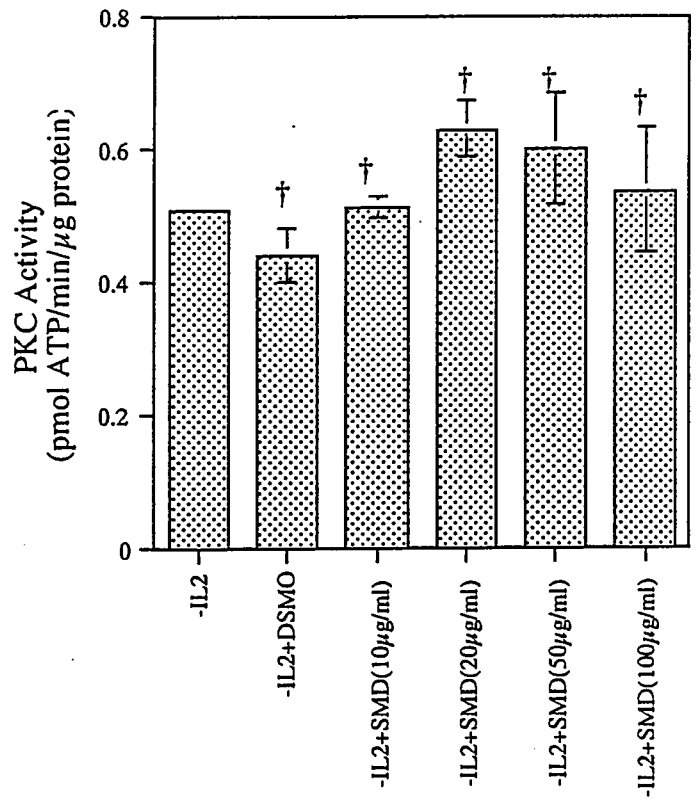
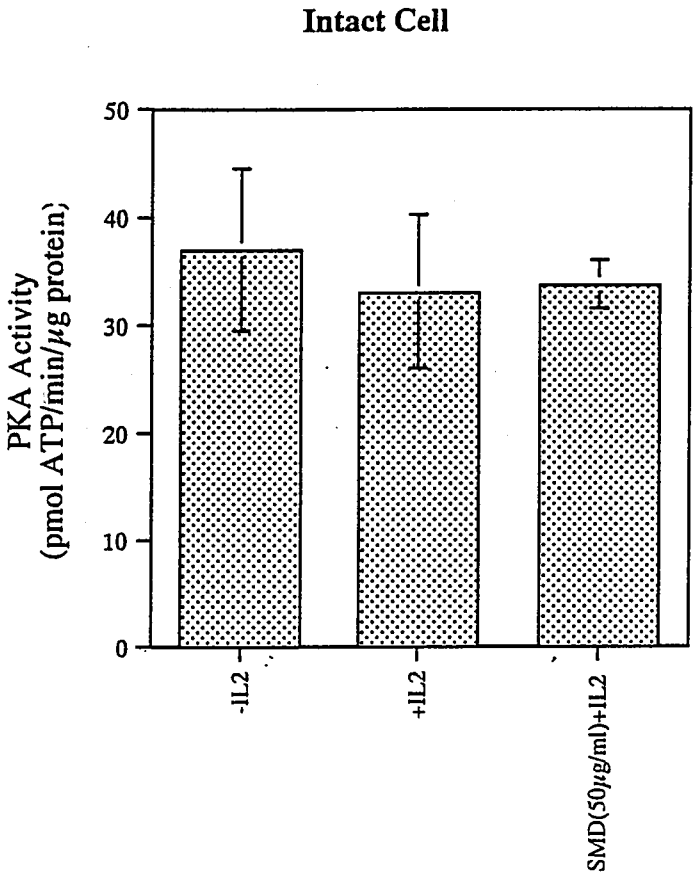


Cell Lysate

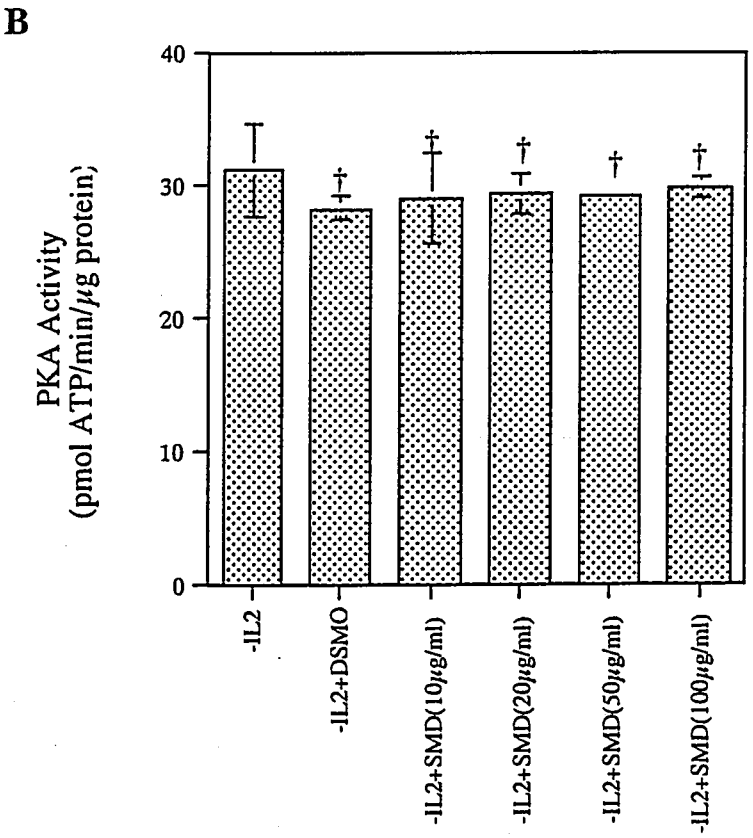
B



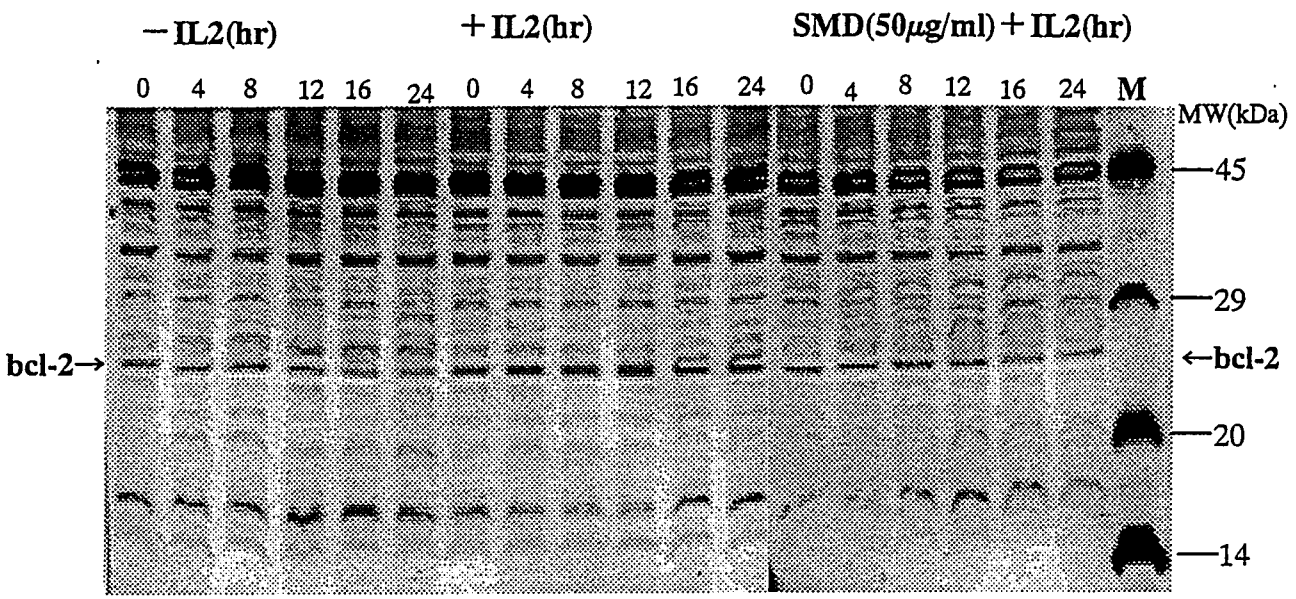
A


Intact Cell
PKA Activity
(pmol ATP/min/μg protein)
50
40
30
20
10
0
-IL2
+IL2
SMD(50μg/ml)+IL2

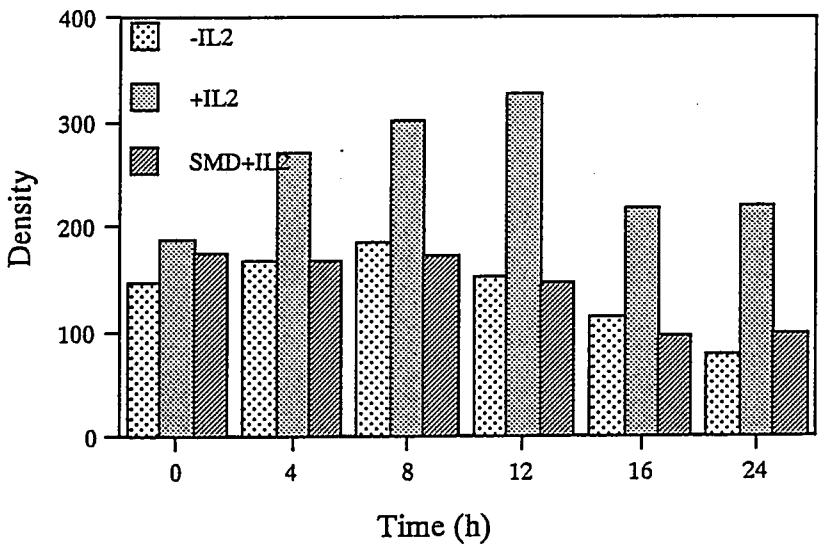
B


Cell Lysate
PKA Activity
(pmol ATP/min/μg protein)
40
30
20
10
0
-IL2
-IL2+DSMO
-IL2+SMD(10μg/ml)
-IL2+SMD(20μg/ml)
-IL2+SMD(50μg/ml)
-IL2+SMD(100μg/ml)

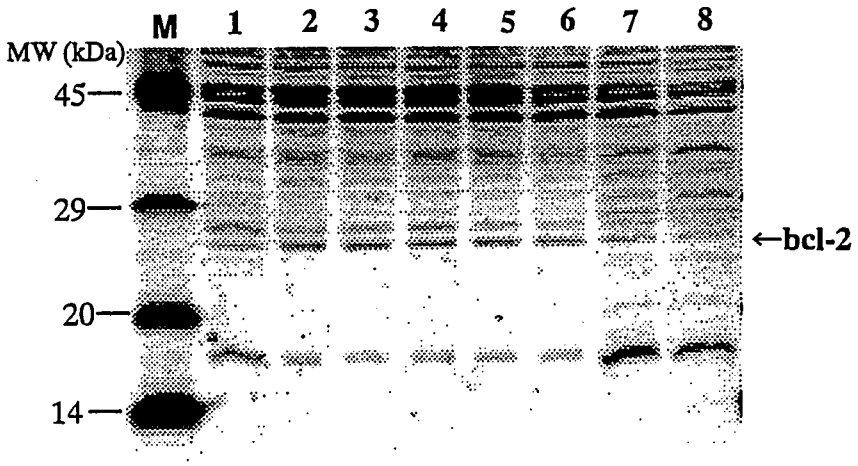
A


−IL2(hr)
+IL2(hr)
SMD(50μg/ml)+IL2(hr)
0 4 8 12 16 24 0 4 8 12 16 24 0 4 8 12 16 24 M
MW(kDa)
45
29
bcl-2→
←bcl-2
20
14

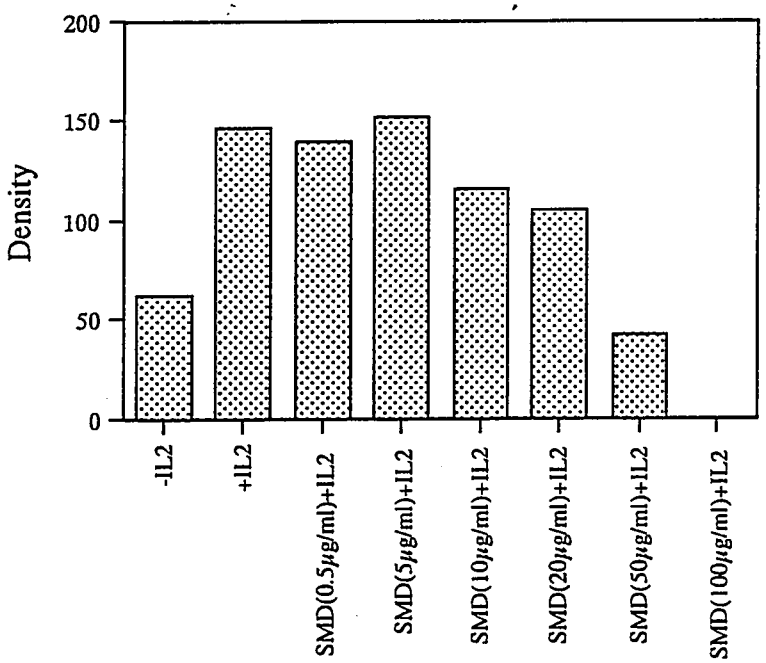
B


400
300
200
100
0
Density
-IL2
+IL2
SMD+IL2
0 4 8 12 16 24
Time (h)

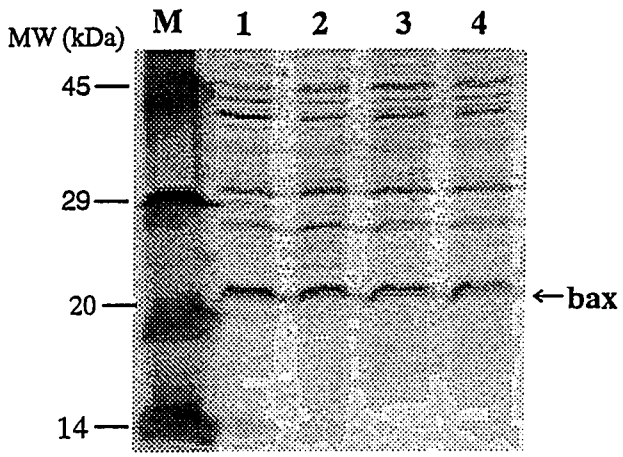
A


M
1
2
3
4
5
6
7
8
MW (kDa)
45
29
20
14
←bcl-2

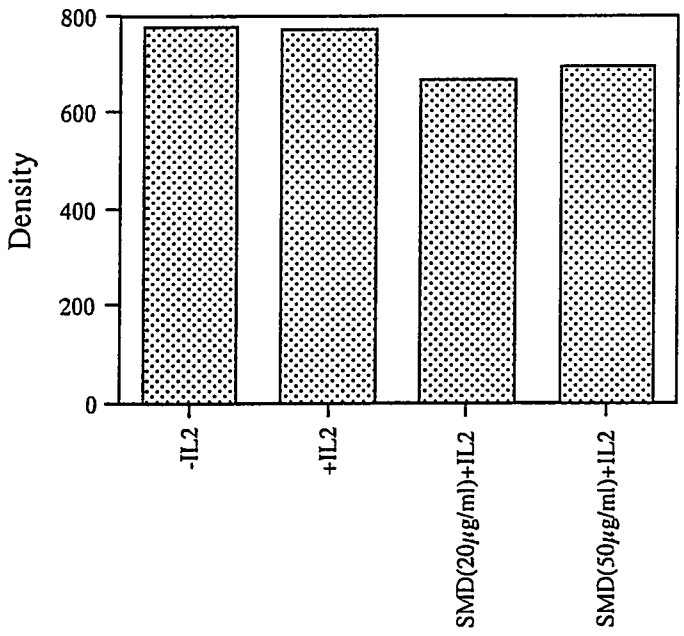
B


Density
200
150
100
50
0
-IL2
+IL2
SMD(0.5μg/ml)+IL2
SMD(5μg/ml)+IL2
SMD(10μg/ml)+IL2
SMD(20μg/ml)+IL2
SMD(50μg/ml)+IL2
SMD(100μg/ml)+IL2

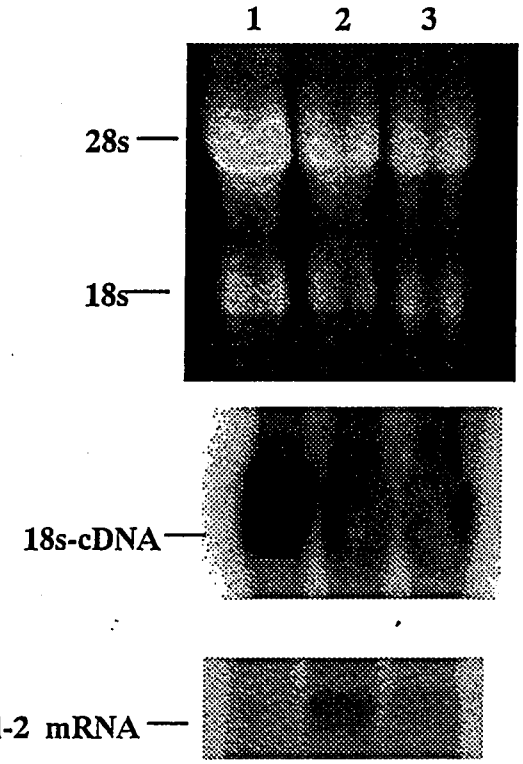
A


MW (kDa)
M
1
2
3
4
45
29
20
14
←bax

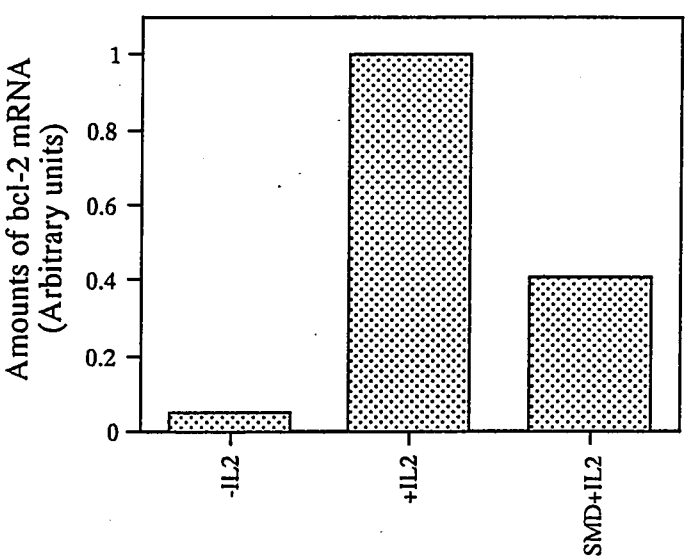
B

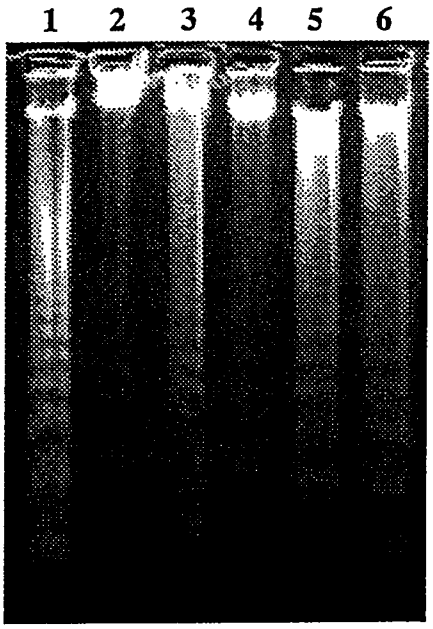

Density
800
600
400
200
0
-IL2
+IL2
SMD(20μg/ml)+IL2
SMD(50μg/ml)+IL2

A

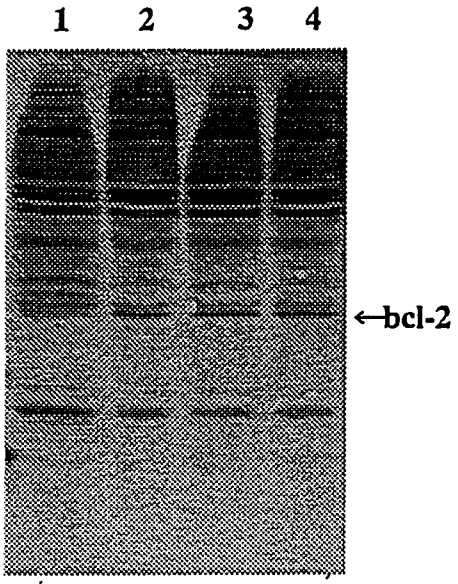

1
2
3
28s
18s
18s-cDNA
bcl-2 mRNA

B

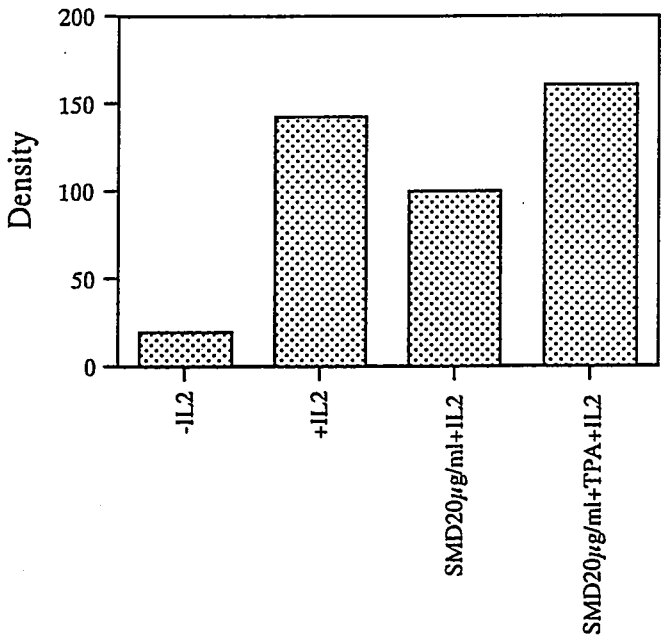

Amounts of bcl-2 mRNA
(Arbitrary units)
0
0.2
0.4
0.6
0.8
1
-IL2
+IL2
SMD+IL2

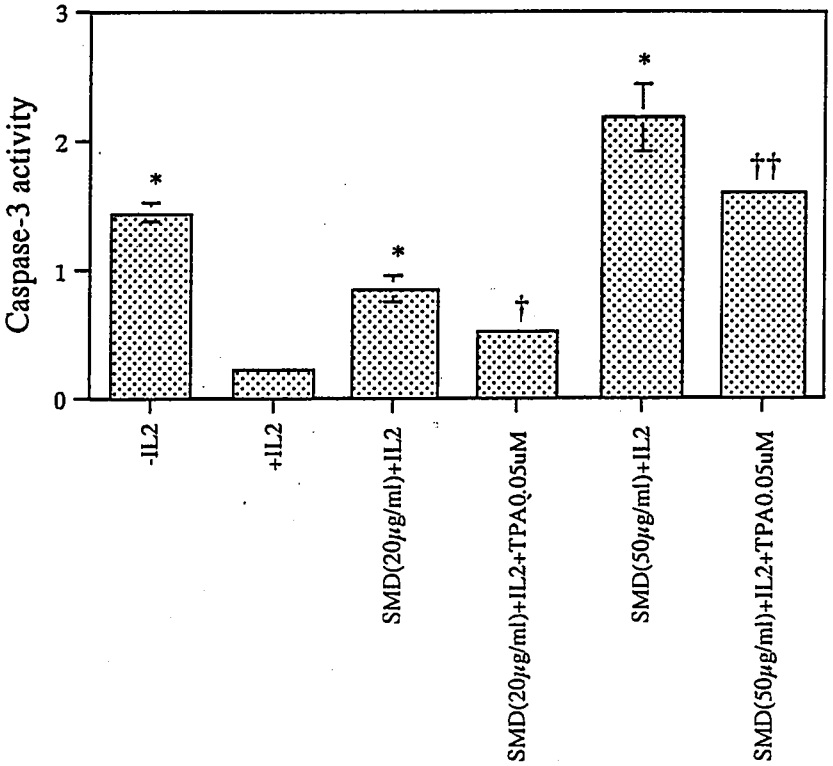

1
2
3
4
5
6

A


1
2
3
4
←bcl-2

B


Density
200
150
100
50
0
-IL2
+IL2
SMD20μg/ml+IL2
SMD20μg/ml+TPA+IL2


Caspase-3 activity
3
2
1
0
*
*
*
†
††
-IL2
+IL2
SMD(20μg/ml)+IL2
SMD(20μg/ml)+IL2+TPA0.05uM
SMD(50μg/ml)+IL2
SMD(50μg/ml)+IL2+TPA0.05uM