

Effects of fibronectin and type IV collagen on osteosarcoma cell apoptosis

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The aims of this study are the investigation of the effects of fibronectin and type IV collagen extracellular matrix proteins and the role of caspase-3 and -9 on cis-platin induced U2-OS apoptosis were studied. First the cytotoxic effects of cis-platin on cell system were investigated by colorimetric method and than morphological and ELISA analysis were used for determination of cell apoptosis when induced with cis-platin. In addition, after adhering the cells to fibronectin or type IV collagen proteins, the apoptotic rate and the effects of caspase-3 and -9 were also investigated by ELISA in presence of specific inhibitors. U2-OS cells showed 20% cytotoxicity after treatment with 2.4 μ M of cis-platin for 48 h. Morphological and the numerical data showed that cis-platin was able to induced apoptosis on cells as a dose-dependent manner. Caspase-3 and -9 inhibitors inhibited cis-platin-induced apoptosis in U2-OS cells, respectively. The binding of cells to 10 μ g/mL of fibronectin but not type IV collagen enhanced the apoptosis about 2.5 fold that effects inhibited with caspase-3 inhibitor. The caspase-3 and -9 are involved in the apoptotic signals induced by cis-platin in U2-OS. The binding to fibronectin, but not type IV collagen enhanced the apoptotic response of U2-OS and fibronectin-dependent apoptosis was activated by caspase-3. These finding might be useful for patients to fight against osteosarcoma.

Keywords: Apoptosis, Cell culture, Fibronectin, Osteosarcoma, Type IV collagen

Osteosarcoma is the most common primary malignancy of the skeletal system, affecting children and young adults¹. This type of malignant tumor is characterized by aggressive invasion, early metastasis and resistance to existing chemotherapeutic agents². Therefore, the current strategy for the treatment of high-grade osteosarcoma is based on surgery and neoadjuvant chemotherapy³. The surgery combined with chemotherapy has markedly improved patient survival (55-70%) during the last years⁴. In addition, the clinical data showed that high doses of methotrexate⁵ and conventional doses of cis-platin, adriamycin and ifosfamide⁶ achieved the best results in the treatment of osteosarcoma patients. Especially, cis-platin is still one of the most effective and commonly used chemotherapeutic agents against osteosarcoma due to its therapeutic advantages, such as high efficiency, mild side effects and easy administration. The mechanism underlying cis-platin anticancer activity is incompletely defined, but it is generally accepted that cis-platin causes DNA damage by forming DNA-DNA or DNA-protein

adducts that trigger cell apoptosis⁷. Mechanisms that activate caspases-8 and caspase-9 or executioner caspase-3 are all known to be involved in cisplatin-induced tubular cell apoptosis and caspase inhibition markedly reduces kidney injury^{8,9}. However, cis-platin resistance often occurs in clinical practice¹⁰. Therefore, major efforts have been made to identify alternative approaches to current chemotherapy¹¹. The available evidence suggests that the molecular profile may affect biological response of tumor cells, thereby suggesting to design combined therapies based on the disease molecular background.

Tumor cell metastasis is a complex process involving the activities of both, tumor cells and host cells. One of the key steps in the metastatic cascade involves the disruption of the extracellular matrix (ECM) and basement membranes, permitting tumor cells to access a distant metastatic site. The interaction between ECM proteins and tumor cell surface receptors is an important initial step in the invasion process. Prior to invasion of the ECM, clonal expansion, growth, diversification and angiogenesis must occur in order to produce metastatic subclones within the primary tumor¹². These transformed tumor cells must then separate from the primary tumor and interact with the ECM at several stages in the

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metastatic cascade. Integrins are the most important family of cell-surface adhesion molecules that mediate interactions between the cells and the ECM. Thus integrins send anchorage-dependent signals that can influence cell survival by suppressing¹³ or inducing apoptosis dependent on cell type. Tumor cells resist detachment-induced apoptosis (anoikis) by establishing contacts with other tumor cells or with host cells such as platelets and inflammatory cells. Both types of interactions generate intracellular signals that prevent anoikis¹².

Based on the known effects of ECM on tumor cell apoptosis, it was thought to evaluate the effects of various ECM proteins (fibronectin or type IV collagen) on osteosarcoma (U2-OS) cell apoptosis. To address this question cell adhesion and apoptosis experiments were carried out with human osteosarcoma cell line U2-OS. Apoptosis was induced by cis-platin because cis-platin is still one of the most effective and commonly used chemotherapeutic agents against osteosarcoma.

Materials and Methods

Cell culture—Cell line of human osteogenic sarcoma (U2-OS) was obtained from American Tissue Culture Collection (ATCC). The cells were cultured in 1964 from a biopsy of a moderately differentiated osteosarcoma obtained from the tibia of a 15-year old Caucasian girl^{14,15}. Cells were grown in McCoy's 5a containing 10% fetal calf serum, 1% penicillin/streptomycin and 1% L-glutamine (GIBCO).

MTT dye reduction assay—U2-OS cells in exponential growth phase were harvested and aliquots (100 μ L) of the cell suspensions were placed into a 96 well microtitre plate. The initial row of 4 wells was filled with 100 μ L of medium alone to serve as a blank and other 4 wells were filled with 100 μ L of cell suspensions without cis-platin to serve as a drug control. Various dilutions of cis-platin (19.2, 9.6, 4.8, 2.4, 1.2, 0.6, 0.3, 0.15, 0.075, 0.0375 μ M) was added to cells and plates were incubated for 24, 48 or 72 h in a 5% CO₂ atmosphere at 37 °C. MTT (3-(4,5-dimethylthiazolyl-2)-2,5 - diphenyltetrazolium bromide) (20 μ L) dye solution (5 mg/mL in sterile PBS) was added and incubated for 2 h. The tetrazolium salt was then solubilised by addition of 200 μ L of DMSO. Absorbance at 540 nm was determined by use of a EL \times 808-IU Bio-Tek plate reader. Values obtained from the cells without cis-

platin were taken as a 100% cell viability. Each drug concentration was repeated 4 times per experiment which was repeated 3 times.

Acridine orange (AO)-ethidium bromide (EB) double staining cell morphological analysis—U2-OS cells (2×10^5) were seeded into each well of a 24-well plate containing cover-slip and incubated for 24 h at 37 °C in a humidified, 5% CO₂ atmosphere. After 24 h, 38.4, 19.2 or 2.4 μ M of cis-platin were added and incubated for 48 h. After incubation, the mixture of acridine orange (50 μ g/mL) and ethidium bromide (50 μ g/mL) was added. Each slide were viewed under a fluorescence microscope (Leica, Germany) with 100 \times magnification.

Detection of apoptosis by ELISA—Cells (1×10^4) were seeded into multiplate well with 2.4 μ M cis-platin. After the incubation of 48 h, the supernatant (containing DNA from necrotic cells that leaked through the membrane during incubation) was discarded. Lysis buffer was added and incubated for 30 min at room temperature. After centrifugation, an aliquot of the supernatant was transferred to a streptavidin-coated well of a microplate. Supernatant was incubated with immunoreagent (containing anti-histone and anti-DNA (peroxidase-conjugated) monoclonal antibodies) for 2 h. The amount of the colored product was determined by spectrophotometrically at 450 nm. The absorbance data of control cells was taken as 100% cell viability. To determination of caspase inhibition, the cells were pretreated with various concentrations of caspase-3 and -9 inhibitors (Ac-Asp-Glu-Val-Asp-CHO and Z-Leu-Glu(OMe)-His-Asp(OMe)-CH₂F, respectively, Calbiochem) for 1 h at 37 °C.

Flow cytometry—U2-OS cells were harvested with 0.25% trypsin/EDTA, resuspended in complete medium and washed twice with cells wash buffer (0.1% Bovine Serum Albumine, BSA). Cell suspensions containing 2×10^5 cells/50 μ L were added to each well and 50 μ L of control or experimental antibodies were also added. After a 30 min incubation at 4 °C, cells were resuspended in wash buffer containing FITC-RAM (DAKO). Cells were resuspended in 10% (v/v) formaldehyde in Phosphate Buffer Saline (PBS). PBS (400 μ L) was added to each well and analysed with a flow cytometer (FACScan; Becton Dickinson).

Cell adhesion assay—96-well plates (BD, Falcon nontreated 96 well-plates, Cat no 351190) were coated with a series concentrations (4, 6, 8, 10 and

15 $\mu\text{g}/\text{mL}$) of fibronectin, type IV collagen or Poly L-lysine as a negative control at 4 °C overnight¹⁶. Non-specific binding sites were blocked with 1% BSA at 4°C for 4 h. Cells (3.5×10^5) were resuspended in serum-free medium containing 2 mM Ca^{2+} and 0.5 mM Mg^{2+} (adhesion buffer). U2-OS cells (100 μL) were added and allowed to adhere to the substrate for 1 h at 37 °C. Unattached cells were removed by washing with adhesion buffer. Adherent cells were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet. A_{550} was measured by a microplate reader. The adherence of U2-OS cells was calculated by subtracting absorbance of blank wells from that of cell lysates.

Statistical analysis—All values were expressed as mean \pm SE. Graph Pad Prism Software was used for statistical analysis. $P < 0.05$ was considered to be statistically significant.

Results

Cytotoxic effects of cis-platin on U2-OS cells—The concentration-effect curve obtained from MTT assay of the cis-platin in U2-OS cells after exposure for 24, 48 and 72 h is shown in Fig. 1. Incubation with cis-platin for 24 h provided very low cytotoxicity in cells treated up to 19.2 μM of cis-platin with a slightly decrement of viability. The cytotoxicity of cis-platin in cells was increased after 48 h of incubation. Higher concentration of cis-platin (19.2 μM) showed 86% cytotoxicity ($P < 0.05$). However the cells treated with lower doses (9.6 μM –0.0375 μM) of cis-platin for 48 h provided cytotoxicity between 90–14% and IC_{50} value was

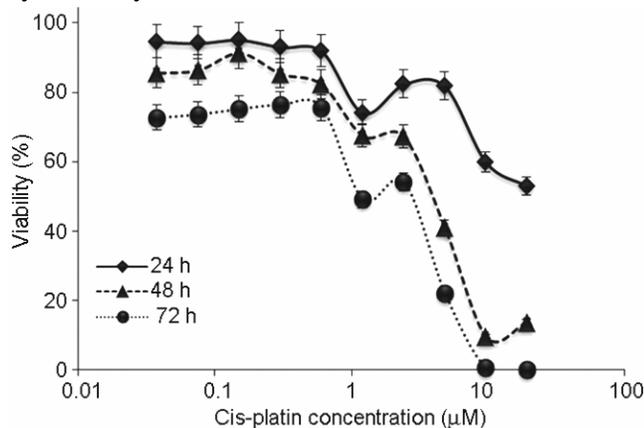


Fig. 1—The effects of cis-platin on U2-OS viability was determined by MTT assay. Pre-determined cell number was incubated with various concentrations of cis-platin for 24, 48 or 72 h at 37 °C. Each bar represents the mean \pm S.D of experiments performed in quadruplicate wells. * $P < 0.05$ represent significant differences between the experimental values.

determined at 4 μM after 48 h of incubation. Further, incubation time (72 h) also increased the cytotoxic ability of cis-platin in cells (cytotoxic range between 100–27%). The cells showed 20% cytotoxicity after treatment with 2.4 μM of cis-platin for 48 h therefore this non-toxic concentration and time was chosen for use in other assays in this study.

Acridine orange (AO)-ethidium bromide (EB) double staining cell morphological analysis (Fig. 2)—U2-OS cells were either untreated with cis-platin or treated with various concentrations of cis-platin for 48 h. Viable cells with intact DNA and nucleus give a round and green nuclei. Early apoptotic cells had fragmented DNA which gives several green colored nuclei. Late apoptotic and necrotic cells DNA were fragmented and stained orange and red. From the data it was clear that with increasing concentration of

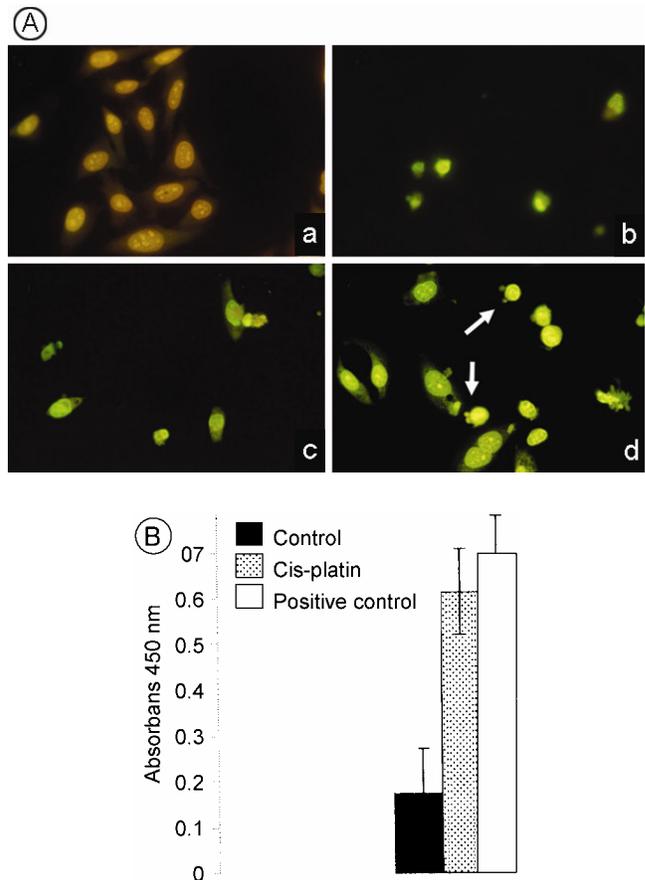


Fig. 2—Image of cis-platin treated U2-OS after acridine orange/ethidium bromide staining (A) and the apoptotic rate of cells by Cell Death Detection Assay after 48 h (B). (A) (a) control cells, (b) cells treated with 38.4 μM cis-platin, (c) 19.2 μM cis-platin (d) 2.4 μM cis-platin for 48 h. The arrows in (d) show early apoptotic nuclei with chromatin super-aggregation and membrane blebbing. 100 \times magnification.

cis-platin, the number of viable cells decreased. Besides, the cells treated with 2.4 μM cis-platin exhibited typical characteristics of apoptotic cells like plasma membrane blebbing (Fig. 2A-d). However the necrotic cells were not appeared. This shows that most of the U2-OS cells were not undergoing necrosis and cell death occurred early through apoptosis.

In addition to morphological analysis, the apoptotic rate was also investigated by using cell death detection assay after the cells were treated with 2.4 μM cis-platin for 48 h. Cis-platin caused about 85% apoptosis as compared to positive cells that were camptothecin-induced U937 cells for 4 h. However 21% of untreated cells only underwent apoptosis (Fig. 2B) indicating that 2.4 μM cis-platin for 48 h is able to induce apoptotic pathway in this cell line and that effect was effective as a positive control.

Caspase-3 and -9 inhibitors decreases apoptosis induced by cis-platin—To determine whether the activation of caspase-3 and -9 are required for the induction of cell death by cis-platin, U2-OS cells were pretreated with caspase-3 inhibitor Ac-Asp-Glu-Val-Asp-CHO and caspase-9 inhibitor Z-Leu-Glu(OMe)-His-Asp(OMe)-CH₂F. Both inhibitors were able to inhibit cis-platin-stimulated apoptosis in dose-dependent manner (Fig. 3). An inhibitor of caspase-3 protease inhibited cis-platin-induced apoptosis in U2-OS cells at 10 μM , whereas caspase-9 inhibitor was able to inhibit apoptosis at 100 μM concentration

(Fig. 3B). Thus, these data suggest that the induction of caspase-3 and -9 activities are a specific biochemical event that brought about apoptosis-inducing cis-platin in U2-OS cells.

The effects of extracellular matrix on cis-platin induced U2-OS cell apoptosis—To determine the effects of ECM proteins on cis-platin induced U2-OS cells apoptosis, firstly the expression levels of fibronectin receptors (αv , α5 and α4 integrins) and type IV collagen receptors (commonly α3 integrin) were investigated by flow cytometry. As shown in Fig. 4, all exhibited different levels of expression.

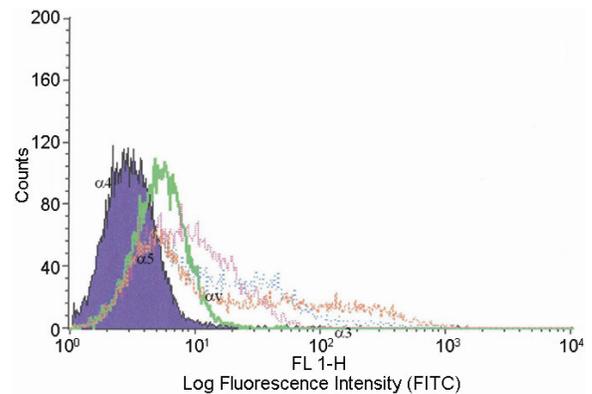


Fig. 4—Flow cytometric histograms of expression of various integrins among the U2- OS cells. Cells were stained with anti- α4 , - α5 , - αv or - α3 integrin antibodies or with FITC conjugated secondary antibodies alone (dark peaks) and subjected to flow cytometric analysis.

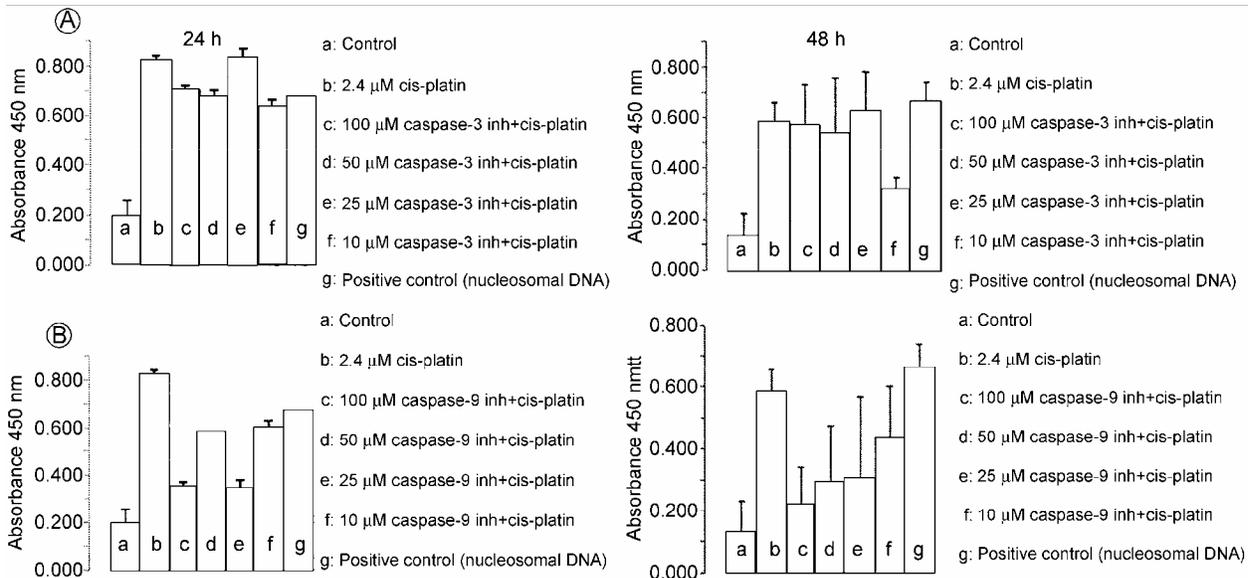


Fig. 3—Inhibition of caspase-3 and -9 activities on U2-OS apoptotic cells by Ac-Asp-Glu-Val-Asp-CHO and Z-Leu-Glu(OMe)-His-Asp(OMe)-CH₂F inhibitors respectively. U2-OS cells were pretreated for 1 h with caspase inhibitors prior to the addition of 2.4 μM cis-platin for 24 and 48 h. The caspase-3 and -9 activity was detected as described under Materials and Methods. Columns represent means for three independent experiments and bar \pm SE.

U2-OS cells showed higher expression level of $\alpha 4$ integrins (M1 - 79%). On the other hand, the expression levels of other integrins were $\alpha v > \alpha 3 > \alpha 5$ (M1 - 48% > 47% > 46%, respectively).

U2-OS cells were examined by adhesion assay to determine the binding capacity of cells to either fibronectin or type IV collagen because U2-OS cells expressed high level of $\alpha 4$, αv and $\alpha 3$ integrins that are fibronectin and collagen IV receptors. Activation of these receptors stimulates the either tumor formation or apoptotic effects upon on cell type. In addition, fibronectin and collagen IV are important ECM proteins for human osteosarcoma cells in bone formation¹⁷. The results (Fig. 5) indicate that the binding of U2-OS cells to fibronectin was increased as a dose-dependent manner whereas the binding of 8 $\mu\text{g}/\text{mL}$ fibronectin was decreased slightly. However the adhesion of U2-OS cells at either 10 or 15 $\mu\text{g}/\text{mL}$ of fibronectin remained unchanged. On the other hand, the binding of U2-OS cells to type IV collagen was more stable than binding to fibronectin. Even the cells showed higher binding capacity at lower concentration of type IV collagen (4 $\mu\text{g}/\text{mL}$). Therefore, 10 $\mu\text{g}/\text{mL}$ of fibronectin and 4 $\mu\text{g}/\text{mL}$ of type IV collagen were selected to be used for other experiments. Pre-determinat expression level and number of integin pattern seemed to be unaffected on U2-OS cell binding or unspecified other type IV collagen integrins could be more effective on cell binding capacity. This is an open area to examine.

The effects of binding of fibronectin and type IV collagen on cis-platin induced U2-OS cell apoptosis were investigated by immunoreagent monoclonal antibodies that binds to apoptotic histone-complexed DNA fragments. Addition of cis-platin to U2-OS cells adhered to cell culture flask increased the number of

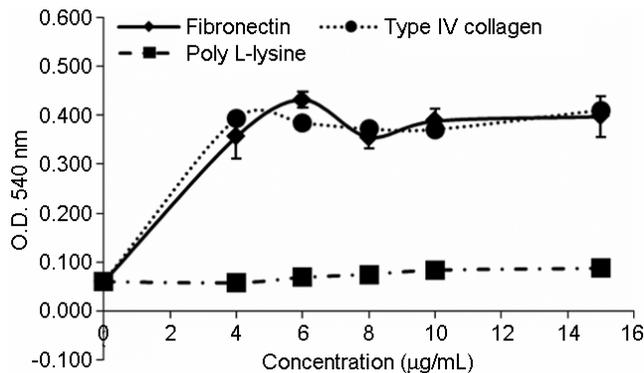


Fig. 5—Binding capacity of U2-OS cells to various ligands was determined by adhesion assay. Each bar represents the mean \pm SD of experiments performed in quadruplicate wells.

apoptotic cells. The absorbance related to the number of apoptotic cells with DNA fragmentation was enhanced about 1.5 fold (Fig. 6). Interestingly, adding of cis-platin to the cells on fibronectin (10 $\mu\text{g}/\text{mL}$) increased the DNA fragmentation about 2.5 fold ($P < 0.01$) (Fig. 6A). The binding of U2-OS cells to fibronectin enhanced the rate of DNA fragmentation as compared to cells bound to culture flask (approximately 1.6 fold). On the other hand, the binding of type IV collagen also increased the apoptotic rate but that rise was not significant as compared to negative control of Poly L-lysine statistically.

Due to the dramatic increase in apoptotic rate of cells adhered to fibronectin and to address the role of caspase-3 on fibronectin-induced U2-OS cell apoptosis, the rate of DNA fragmentation was investigated in presence of caspase-3 inhibitor (Fig. 6B). It was found that pre-treated with 10 μM

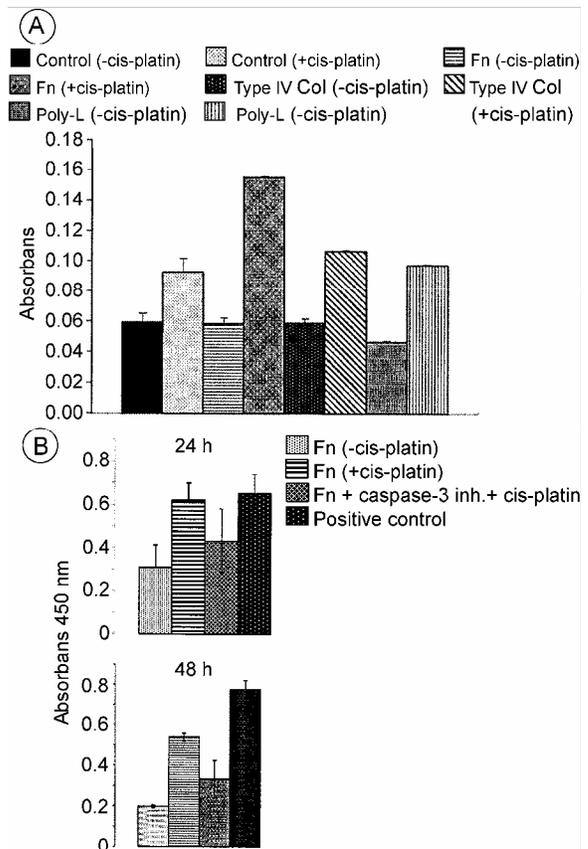


Fig. 6—The apoptotic rate of cis-platin treated U2-OS after adhering to either fibronectin (10 $\mu\text{g}/\text{mL}$) or type IV collagen (4 $\mu\text{g}/\text{mL}$) for 48 h (A) and the role of caspase-3 on cells adhered to Fn for 24 and 48 h (B). The apoptotic rate was determined by enzyme-immunoassay as described in Materials and Methods. Each column represents the mean \pm SD of experiments performed in triplicate wells.

caspase-3 decreased the cis-platin induced DNA fragmentation in U2-OS cells bound to fibronectin as compared to untreated cells (1.6 fold). These inhibition studies have shown that the activation of caspase-3 enzyme is involved in fibronectin-induced U2-OS cell apoptosis.

Discussion

Despite significant therapeutic improvements over the past several decades, osteosarcoma still has a poor prognosis in about 50% of patients¹⁸. Surgery of primary tumour, radiation and chemotherapy are still widely used in treatment of osteosarcoma. Of these, particularly the benefit of chemotherapy has been validated by Multi-Institutional Osteosarcoma Study (MIOS) conducted in the 1980's, in which patients were randomized to surgery alone versus adjuvant chemotherapy following amputation for localized osteosarcoma. The patients randomized to receive chemotherapy had a significant improvement in their survival rate¹⁹. This significant difference persisted at follow-up greater than 5-years, validating that the addition of chemotherapy improves overall survival and does not just delay time to progression. Recently, neoadjuvant chemotherapy has become accepted practice in the majority of centres using protocols which include the most active agents in this disease; doxorubicin, cis-platin and methotrexate. Newer agents, particularly ifosfamide and etoposide, are increasingly incorporated into complex regimens²⁰.

In addition, investigators attempting to identify new agents or combination regimens have attempted to improve the survival rate of osteosarcoma patients. Lately, it has been demonstrated that increasing the number of agents in the neoadjuvant period increases the percentage of patients with higher grades of necrosis²¹. Therefore, in addition to traditional chemotherapy, there are multiple investigational agents such as muramyl tripeptide-phosphatidyl ethanolamine²², interferon α -2 β ²³ and granulocyte-macrophage colony-stimulating factor being studied which target pathways that are believed to be active in osteosarcoma.

Fibronectin is an abundant glycoprotein in the ECM and is present at its highest concentration during osteogenesis. Osteoblasts cells bind to fibronectin preferentially and more strongly than other ECM proteins²⁴. Fibronectin contributes to osteoblastic adhesion, differentiation and matrix mineralization^{25, 26}. Expressed from early stages of differentiation, collagen

is another ECM protein that is essential for bone formation and matrix production²⁷.

Considering the above results, it was thought that the binding of fibronectin and collagen could be also effective on cis-platin induced apoptosis of osteosarcoma and therefore that was used in practice in addition to traditional chemotherapy.

Primarily, the apoptotic rate and the role of caspase-3 and -9 on cis-platin induced U2-OS apoptosis were investigated. The results demonstrated significant enhancement in the rate of apoptosis (85%) after treatment of U2-OS cells with cis-platin for 48 h. After addition of either caspase-3 or caspase-9 inhibitor in culture medium, the rate of cis-platin induced apoptosis on U2-OS cells was also inhibited. Caspase-3 is the major apoptotic executioner protease and directly cleaves most of the proteins that are proteolyzed during apoptosis^{28,29}. Caspase-3 is activated by both apoptotic pathways: an extrinsic pathway that depends on the activation of the death receptor members of the tumor necrosis factor receptor family^{30,31} and an intrinsic mitochondrial pathway controlled by Bcl-2 family proteins^{32,33}. However, caspase-9 is the essential initiation caspase required for apoptosis signalling through the mitochondrial pathway. The inhibition results indicated that cis-platin treatment in U2-OS cells stimulated the intrinsic apoptotic pathway, a finding consistent with caspase-3 assay results (Fig. 3). However still the release of cytochrome c in the cytosol should be examined. Because the release of cytochrome c is widely accepted to be responsible for the activation of caspase-9; once activated, caspase-9 activates caspase-3, which eventually results in the execution of apoptosis³⁴.

The survival of many types of mammalian cells depends on positive signals such as hormones, growth factors, cell-cell and cell-matrix interactions. In normal cells, survival is depending on the appropriate surrounding environment such as cell-cell interaction, cell-matrix interaction, growth factors and hormones action³⁵. Cell-matrix interaction via integrins is essential for cell growth control, cell survival and apoptosis. Inadequate cell-matrix interaction induces an anoikis^{36, 37}. Cis-platin was able to promote apoptosis in U2-OS cells adhered to both fibronectin and type IV collagen (Fig. 6). However the apoptotic rate of cis-platin treated U2-OS cells on fibronectin was increased about 2.5 fold from the cells on type IV collagen, suggesting that fibronectin

enhanced the apoptotic effects of cis-platin on U2-OS cells. The apoptotic response induced by cis-platin in U2-OS cells might have occurred prior to cell detachment. As mentioned above, cell-matrix detachment usually induces an anoikis; however the present results showed that the binding of cis-platin treated U2-OS cells to fibronectin enhanced the apoptotic rate that could be because of high expression of $\alpha 4$ integrin in these cells (Fig. 4). This stimulates cell death resulting in an apoptotic signal with different kinetics than the apoptotic signal induced by matrix detachment³⁸. Cis-platin could cause the integrin-mediated cell death in U2-OS cells. This type of apoptosis is both biologically and biochemically different from anoikis, which is due to cell detachment from substratum. Interestingly, cis-platin induced apoptosis on fibronectin inhibited by caspase-3 inhibitor (Fig. 6B) suggesting that integrin-mediated cell death of U2-OS cells could be involved in caspase-3 activation. Recently it has been reported that integrin-mediated apoptosis induced by recruitment of caspases activities to the integrin subunit tails of unligated integrins in human keratinocytes³⁹. Unlike caspase-9, caspase-8 is involved in extracellular signal-induced apoptosis mediated by specific death receptors such as Fas and TNF-R located at the cell surface⁴⁰. Further studies are needed in order to identify caspase-8 involved in the cis-platin-induced apoptotic response in this cell system.

The present results indicate that caspase-9 is involved in the apoptotic signals induced by cis-platin in U2-OS cell system. However binding of U2-OS cells to fibronectin, but not type IV collagen enhanced the apoptotic response and caspase-3 involved in. The mechanism of fibronectin induced apoptosis in osteosarcoma cells needs to be investigated further.

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