Effect of human granulocyte macrophage-colony stimulating factor on differentiation and apoptosis of the human osteosarcoma cell line SaOS-2

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We investigated the effects of human granulocyte macrophage-colony stimulating factor (GM-CSF) on the relation between differentiation and apoptosis in SaOS-2 cells, an osteoblast-like cell line. To determine the relationship between these cellular processes, SaOS-2 cells were treated *in vitro* for 1, 7 and 14 days with 200 ng/mL GM-CSF and compared with untreated cells. Five nM insulin-like growth factor (IGF-I) and 30 nM okadaic acid were used as negative and positive controls of apoptosis, respectively. Effects on cell differentiation were determined by ECM (extracellular matrix) mineralization, morphology of some typical mature osteoblast differentiation markers, such as osteopontin and sialoprotein II (BSP-II), and production of bone ECM components such as collagen I. The results showed that treatment with GM-CSF caused cell differentiation accompanied by increased production of osteopontin and BSP-II, together with increased ECM deposition and mineralization. Flow cytometric analysis of annexin V and propidium iodide incorporation showed that GM-CSF up-regulated apoptotic cell death of SaOS-2 cells after 14 days of culture in contrast to okadaic acid, which stimulated SaOS-2 apoptosis only during the early period of culture. Endonucleolytic cleavage of genomic DNA, detected by "laddering analysis", confirmed these data. The results suggest that GM-CSF induces osteoblastic differentiation and long-term apoptotic cell death of the SaOS-2 human osteosarcoma cell line, which in turn suggests a possible *in vivo* physiological role for GM-CSF on human osteoblast cells.

Key words: GM-CSF, ostesarcoma cells, apoptosis.

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Human granulocyte macrophage-colony stimulating factor (GM-CSF) is a major cytokine active on hematopoietic cells at different stages (Aglietta et al., 1991; Abbas et al., 1991; Kittler et al., 1992). It induces a large variety of biological effects, such as proliferation and differentiation of myeloid and CD34-positive progenitors (Metcalf, 1989; Nishijima et al., 1995). Recent evidences indicate that GM-CSF is active also on a variety of cell types of non-hematopoietic origin, such as keratinocytes, solid tumor lines, bone marrow fibroblasts and endothelial cells (Dedhar et al., 1988; Colotta et al., 1993; Bussolino et al., 1991). It exerts different effects on normal cell growth and development through a receptor-mediated process (Ruff et al., 1986; Schmitz et al., 1995).

Apoptosis, or physiological cell death, is representative of an endogenous *cell suicide* mechanism which can be selectively triggered by cells in response to stimuli still largely unknown (Willie et al., 1980; Green, 2000). This phenomenon is mediated by various types of proteins, among which there is the Bcl-2 family. This family can be divided into three subgroups. The members of the first subgroup, represented by Bcl-2 and Bcl-XL, have anti-apoptotic function; members of the second and third subgroups, respectively, represented by Bax and Bak, and Bid and Bad, are pro-apoptotic molecules. Apoptosis, furthermore, is mediated by another family of proteins called caspases, which are activated after their inactive precursor has been processed. Caspase-9 and -8 are initiator caspases that are involved in mitochondrial-mediated and death domain-mediated apoptosis, respectively. Caspase-3, -6 and -7 function as effectors of apoptosis (Gottlieb, 2000).

During apoptosis, a series of well-defined degenerative changes occur within the cell (Duvall and Wylie, 1986) and ultimately result in the degradation of the nuclear DNA into oligonucleosome chains (Willie,

1980) and the fragmentation of the cell into neat *bite-size* pieces which can be specifically recognized by macrophages and subsequently phagocytosed (Duvall et al., 1985, Savill et al., 1989, Fadok et al., 1992). Hematopoietic cytokines, such as interleukin-3 (IL-3) and GM-CSF, can prevent apoptosis of hematopoietic cells by activating a specific signalling pathway distinct from that of cell proliferation (Kinoshita et al., 1995). Nevertheless, the effects of GM-CSF on apoptosis of non-hematopoietic cells are not well known (Muto et al., 2001).

A few years ago, Rodan et al. (1987) characterized a new human osteosarcoma cell line, called SaOS-2. Using these cells, a set of properties which seemed to be associated with a well differentiated osteoblastic phenotype, such as elevated levels of alkaline phosphatase and parathyroid hormone-stimulated adenylate cyclase, and synthesis and secretion of type I collagen, was established *in vitro*.

Recent data have shown that the SaOS-2 cell line is able to secrete GM-CSF, leading to questions concerning the biological significance of the presence of this growth factor on the cell membrane, and the presence of the cytokine on the extra-cellular matrix (ECM) (Taichman et al., 1996). In a previous paper, we showed that SaOS-2 cells expressed GM-CSF receptors, and that GM-CSF was able to regulate the proliferation and differentiation of these cells. In fact, during treatment with GM-CSF, SaOS-2 cells increased their production of bone ECM collageneous and non-collageneous components. Furthermore, GM-CSF appeared to be able to regulate, after 14 days of culture, cell differentiation of SaOS-2 cells through stimulation of alkaline phosphatase activity. On the contrary, the effect of the cytokine on cell growth was anti-proliferative (Postiglione et al., 2003).

In the present study, we investigated the effects of GM-CSF on some ECM structures known to be involved in mineralization of bone tissue, such as osteopontin and sialoprotein II (BSP-II), two non-collagenous hydroxyapatite crystal-binding proteins produced mainly during the late stages of osteoblastic differentiation (Kasugai et al., 1992). Furthermore, we investigated the synthesis of collagen I, the major specific component of bone connective tissue produced by osteoblasts during differentiation (Owen et al., 1990). The synthesis of collagen I was also modulated by GM-CSF (Postiglione et al., 2003), which may confirm that SaOS-2 cell differentiation, under GM-CSF stimulation, goes toward a typically *mature* osteoblastic phenotype.

Since it is well known that various types of cells can be induced to undergo apoptosis when they achieve a terminal step of differentiation and maturation (Martin et al., 1990), we analysed the biological effects of GM-CSF on differentiation and apoptosis of the SaOS-2 cells *in vitro*. Our purpose was to investigate whether the treatment with this cytokine can modulate the SaOS-2 apoptotic pathway, probably through inhibition of cell growth, in addition to an influence on their maturation stage.

Insulin-like growth factor (IGF-I), which has an anti-apoptotic effect on SaOS-2 cells (Schmid et al., 1999), was used as a negative control of apoptosis, while okadaic acid, an inhibitor of protein phosphatases 1 and 2A (Shenolikar, 1995), was used as a positive control.

Materials and Methods

Cell culture

The human osteosarcoma cell line SaOS-2 (ATCC85-HTB) was cultured in Dulbecco's modified Eagles medium (DMEM) (Sigma, St. Louis, MO, USA), with 10% heat-inactivated FCS (Gibco, Grand Island, NY, USA), 2 mM L-glutamine, 10 U/mL penicillin and 0.1 mg/mL streptomycin (Sigma) in a humidified incubator at 37°C under an 95% air/5% CO_2 mixture. Medium was changed every 3-4 days. Cells were detached by 0.5 mM EDTA in calcium- and magnesium-free phosphate-buffered saline (PBS) with 0.05 % trypsin.

"In vitro" treatment of cell cultures

Human recombinant GM-CSF (300 µg/mL) was used (Schering Plough, County Cork, IR). SaOS-2 cells were cultured adding 10, 100 and 200 ng/mL of GM-CSF to the culture medium. In other SaOS-2 cell cultures, 30 nM okadaic acid (Sigma) was used as a positive control and 5 nM IGF-1 (Sigma) was used as a negative control of apoptosis.

Freshly prepared amounts of GM-CSF, at the same concentrations, were added every three days.

Cytochemistry

For cytochemical analysis, GM-CSF-stimulated and unstimulated SaOS-2 cells were plated, grown on glass coverslips, and stained with the Von Kossa method modified for cell culture (Postiglione et al., 2003) after 1, 7 and 14 days of culture. Briefly, cells were fixed for 3 min with 3% formaldehyde in PBS, washed twice in PBS, covered with a 0.5% aqueous

solution of silver nitrate and exposed to UV light (Philips, 30W) for 30 min at 25°C. Coverslips were then washed with distilled water and treated with 5% Na_2SO_4. After washing in tap water, coverslips were covered with 1% neutral red to stain nuclei and then submitted to the routine passages in alcohol, xylene and mounting medium.

All microscope evaluations were made on a LEICA DM LB equipped for microphotography by three independent observers and expressed on a five points arbitrary scale, ranging from negative (0) to very abundant (++++, i.e. over 60% of the cells).

Immunocytochemistry

GM-CSF-stimulated and unstimulated SaOS-2 cells were plated and grown on glass coverslips and fixed after 1, 7 and 14 days of culture with 3% formaldehyde in PBS for 3 min at room temperature. The cells were then gently washed twice with PBS and incubated with monoclonal IgG antibodies against human osteopontin or BSP-II (University of Iowa, Iowa City, USA), or with monoclonal anti-human collagen I (Sigma). After three further washes with PBS, the cells were stained with rhodamine or fluorescein-labeled rabbit anti-mouse IgG. The same coverslips were contemporaneously incubated with fluorescein or rhodamine-tagged phalloidin, in order to delineate cell morphology by actin visualization. Both steps required 1 hour incubation at 37°C in a humidified chamber. Coverslips were then mounted with Moviol and observed with a LEICA DM LB equipped for epifluorescence and microphotography. When needed, the coverslips were treated with Triton X100 buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X100, pH 7.4) in order to permeabilize the cells before the primary antibody step, and then processed as described (Polak and Van Noorden, 1992). Each sample was evaluated by three independent observers, using a four point arbitrary scale, ranging from 0 to 3.

Flow cytometric analysis

SaOS-2 cells were plated in 60 mm Petri dishes at different density ($10x10^3$ at 1 day; $70x10^3$ at 7 days; $30x10^3$ at 14 days) to ensure a confluence of 75% of the cells for each experimental point. Cells were harvested by trypsinization and centrifuged at 1500 rpm for 5 min. The pellet was stained with the apoptotic detection kit Annexin-V-FLUOS (Roche, Mannheim, Germany). This method is based on the simultaneous staining of cell surface phosphatidylserine (with Annexin-V-FLUOS-green dye), and necrotic cells (with propidium iodide). Exclusion of propidium iodide, coupled with binding of Annexin-V-FLUOS, indicates the percentage of apoptotic cells. The procedure involved the staining of the pelleted cells in a solution containing Annexin-V-FLUOS and propidium iodide and an incubation of 15 min at room temperature. Cells were then washed twice in PBS and analysed by flow cytometry using a FACScan (Becton Dickinson, Mountain View, Ca, USA) at wavelengths of 530-570 nm. Flow cytometric measurement of 100,000 events was performed and apoptotic analysis was executed by CELL LYSIS software.

For intracytoplasmic Bcl-2 detection, the Fix and Perm kit (Caltag, Burlingame, CA) was used. This procedure gives to the antibodies access to intracellular structures, preserving at the same time the morphological scatter characteristics of cells. The method involves a sequential reaction with a fixing solution followed by a permeabilizating one; finally, direct immunofluorescence using a monoclonal FITC antibody (mAb) against Bcl-2 (Caltag) was performed. Nonspecific anti-mouse FITC IgG was used as a control (Sigma). The fluorescence intensity of the cell suspensions was measured by flow cytometry using a FACScan (Becton Dickinson) at the wavelengths of 530-570 nm and the mean fluorescence intensity values on the scatter dot plot of the apoptotic and non-apoptotic cell populations were evaluated. Each experiment was repeated three times.

DNA isolation and electrophoresis

Cells were lysed in 0.5% Triton X100, 40 mM Tris, pH 7.4 and 0.1 mM EDTA. After spinning in a microfuge at 4°C for 20 min, the soluble fraction was extracted twice with an equal volume of a 1:1 phenol:chloroform mixture. Soluble DNA was precipitated by the addition of 2.5 volumes of ethanol and 300 mM NaCl. DNA was separated on a 1.5% agarose gel.

Statistical analysis

The difference between the mean intensity results of the flow cytometric analysis of treated and untreated cells was analyzed by the Student's t-test for paired samples. Results were considered significant at $p < 0.05$.

Results

Cytochemistry

The deposition of calcified ECM by SaOS2 cells as determined by the Von Kossa method clearly demonstrated an increase of calcium precipitates in the ECM: this effect was evident as early as 72 hours in culture (Table 1) and became more evident during the following days at high cytokine concentrations. In some samples, the deposition of calcium precipitates strictly adherent to the cell membrane of SaOS-2 cells became evident after one week in culture at the highest GM-CSF concentration (Figure 1).

Table 1. Calcium deposition in GM-CSF-treated SaOS-2 cells. The Von Kossa method demonstrates an increase in calcium precipitates in the ECM which becomes more evident at longer times of culture at high concentrations of the cytokine.

Time of treatment	*Control*	*GM-CSF (10 ng/mL)*	*GM-CSF (100 ng/mL)*	*GM-CSF (200 ng/mL)*
24 h	++	+	++	++
7 d	++	+++	+++	++++
14 d	+++	+++	++++	+++++

Immunocytochemistry

Immunofluorescence analysis demonstrated that GM-CSF was able to induce the expression of mineralization markers in SaOS-2 cells. It is interesting to note that osteopontin became evident very early in some SaOS-2 cells treated with high concentrations of GM-CSF. However, while in the early days of culture the marker appeared both in the cytoplasm and on the membrane, after a few days it was expressed mainly on the cell membrane (Figure 2, A and B). The GM-CSF-induced differentiation of SaOS-2 cells in a *more mature* phenotype was confirmed by the concentration- and time-dependent increases in the synthesis of BSP-II, a non-collagenous protein, involved in bone matrix deposition (Figure 2, C and D).

As regards the production of collagen I, morpho-

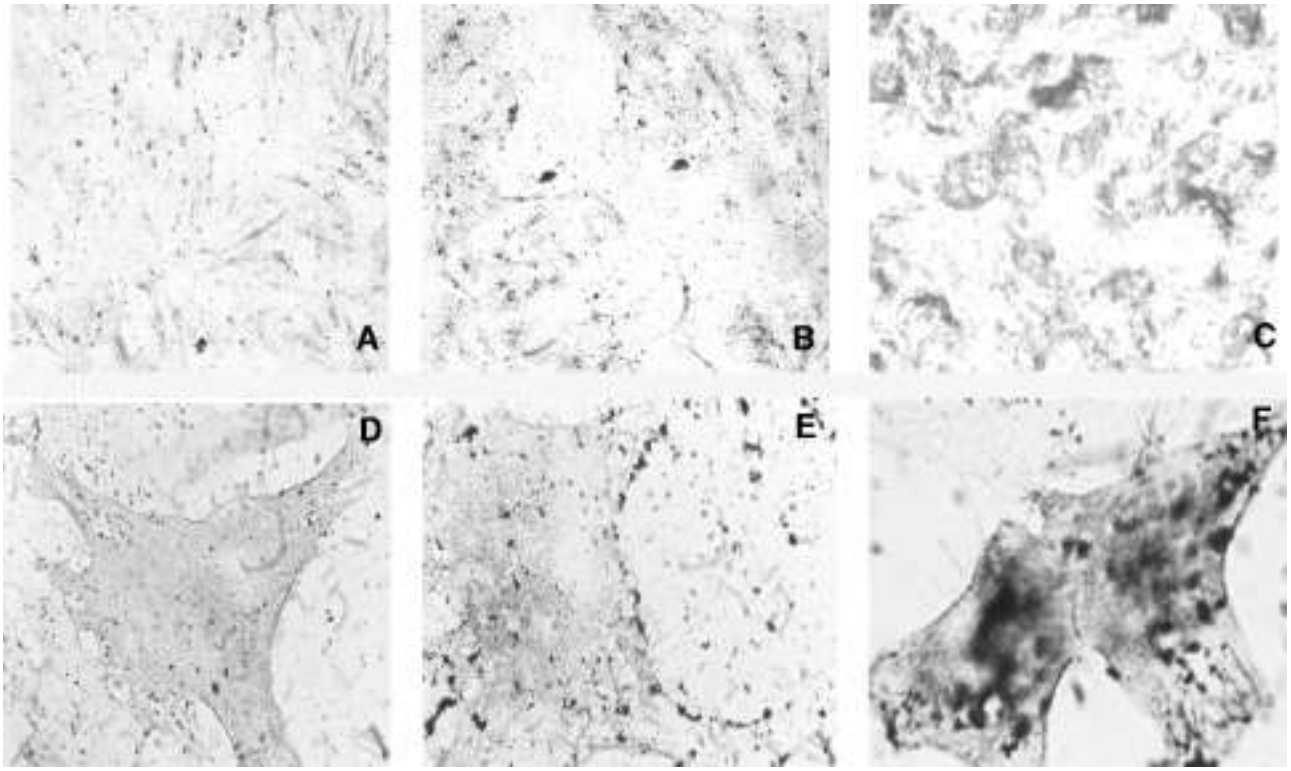

Figure 1. Effects of GM-CSF on ECM mineralization. Stimulated and unstimulated SaOS-2 cells were stained by the Von Kossa method modified for cell culture. After 14 days of culture, the Von Kossa staining evidences: A) a light deposition of calcium in basal conditions; B) enhanced presence of calcium precipitates in SaOS-2 cultures treated with 100 ng/mL of GM-CSF and C) a further increase in calcium deposition in SaOS-2 cultures treated with 200 ng/mL of GM-CSF. D), E) and F) are more detailed pictures from similar fields. As evidenced, Von Kossa-positive granules are more abundant with increasing concentration of GM-CSF. A-C) Magnification 100X; D-F) Magnification 1000X.

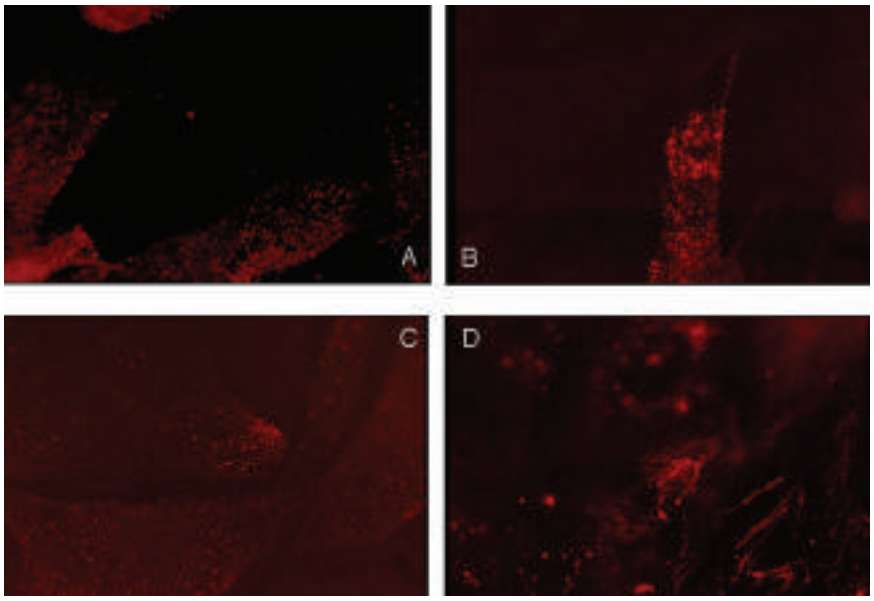

Figure 2. Effects of GM-CSF on the synthesis of non-collagenous protein involved in bone deposition. Immunocytochemistry demonstrates that GM-CSF increased the expression of mineralization markers such as osteopontin (A, untreated and B, treated SaOS-2 cells) and BSP-II, (C, untreated and D, treated cells). Magnification 1000X

logical analysis demonstrated the presence of collagen fibers (Figure 3) around both the unstimulated and the GM-CSF treated cells, with an increase during the last period of culture after GM-CSF addition.

Flow cytometric analysis

To determine whether GM-CSF can affect cell apoptosis of SaOS-2, we examined these cells by flow cytometric analysis after 1, 7 and 14 days of culture, after treatment with 200 ng/mL of GM-CSF. No significant difference was found either in the GM-CSF- or the IGF-1-stimulated SaOS-2 cells after 1 and 7 days of culture, as compared to unstimulated ones ($p > 0.05$). Moreover, GM-CSF significantly doubled annexin V incorporation ($p < 0.03$) and propidium iodide ($p < 0.04$) staining after 14 days of culture, while no significant difference was found in annexin-V incorporation induced by IGF-I, as compared with the unstimulated cells ($p > 0.05$) (Table 2).

Okadaic acid induced a statistically significant 2-5 fold increase in cell apoptosis when compared to the unstimulated cells after 7 ($p < 0.02$) and 14 ($p < 0.03$) days of culture (Table 2).

In order to determine if apoptosis of SaOS-2 cells was mediated by Bcl-2 down-regulation, intracytoplasmatic Bcl-2 protein was measured. Only minimal differences in the mean fluorescence intensity of Bcl-2 protein were observed, which were not statistically significant ($p > 0.05$) in the SaOS-2 cells treated with GM-CSF, IGF-1 or okadaic acid, as

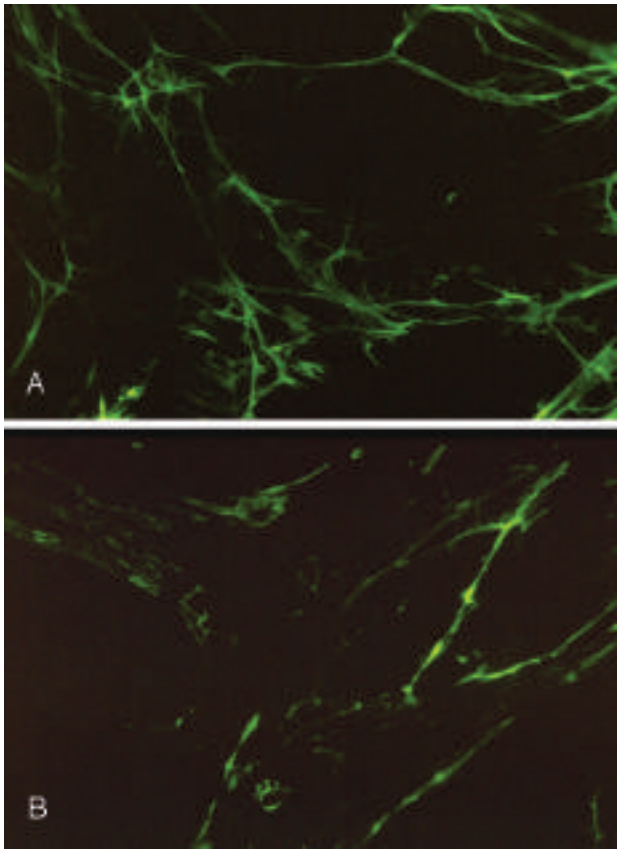

Figure 3. Effects of GM-CSF on SaOS-2 production of collagen I, another bone ECM component: the presence of collagen fibers is more evident in GM-CSF treated cells, with an increase after 14 days of culture (A) when compared with unstimulated cells (B). Magnification 1000X

Table 2. Flow cytometric analysis of apoptosis in SaOS-2 cells. Values are represented as Mean Intensity ± SD at each time point of three independent experiments. The percentage of expression is shown in parentheses.

	1 day			*7 days*			*14 days*		
Treatment	*P.I.*	*Ann. V*	*Bcl-2*	*P.I.*	*Ann.V*	*Bcl-2*	*P.I.*	*Ann.V*	*Bcl-2*
Unstimulated	1197 ±10 (13%)	2053±20 (14%)	182 ±10 (50%)	3081±20 (50%)	228 ±10 (2%)	243±10 (50%)	1336 ±14 (19%)	111 ±7 (4%)	241±10 (54%)
+IGF (15 nM)	847±15 (9%)	2378±25 (15%)	168±15 (44%)	2026±18 (18%)	251±10 (2%)	231±15 (55%)	1214±19 (7%)	180±5 (5%)	229±15 (57%)
+Okadaic acid (30 nM)	1430±15 (15%)	3465±30 (27%)	180±7 (50%)	1961±20 (45%)	1435±15* (47%)	337±20 (50%)	3115±20*(45%)	631±10*(43%)	256±19(44%)
+GM-CSF (200 ng/mL)	1360±18 (14%)	1655±20 (9%)	179±10 (48%)	3008±25 (50%)	223±10 (2%)	218±10 (44%)	2873±20* (39%)	242±5*(8%)	210±10 (50%)

**p<0.05 versus the unstimulated SaOS-2 cells. No significant difference was found either in GM-CSF or IGF-1 stimulated and unstimulated SaOS-2 cells after 1 and 7 days of culture. GM-CSF doubled the annexin V incorporation and propidium iodide staining after 14 days of culture, while no significant difference was found in IGF-I induced annexin-V incorporation. Okadaic acid induced an increase of cell apoptosis between 2-5 fold after 7 and 14 days of culture. Furthermore, treatment of SaOS-2 cells with GM-CSF, IGF-1 and okadaic acid produced no statistically significant difference in Bcl-2 protein expression during the whole period of culture.*

compared to untreated cells. These results were noticed both for the apoptotic cells and the non-apoptotic cell fractions during the entire period of culture (Table 2).

Electrophoretic detection of DNA fragmentation

After 14 days of culture, GM-CSF and okadaic acid generated internucleosomal cleavage which formed a ladder DNA fragment, as is characteristic in cells undergoing programmed cell death. In contrast, only minimal DNA degradation products were detected in unstimulated SaOS-2 cells, or in IGF-I treated cultures (Figure 4).

Discussion

It is well known that GM-CSF has a variety of biological roles in both normal and tumor cells (Foulke et al., 1990) and previous studies demonstrated that it can suppress apoptosis in hematopoietic cells by activating a signalling pathway distinct from the induction of DNA synthesis (Kinsohita et al, 1995).

We recently reported that the human osteosarcoma SaOS-2 cell line expresses GM-CSF receptor and that GM-CSF can modulate cell differentiation of these cells, probably through the inhibition of cell growth (Postiglione et al, 2003). Several studies have demonstrated a sequence of growth, differentiation, maturation and apoptosis in human cells of different embryonic origin (Robbins et al., 1997; Le et al., 2002).

In this report, we examined whether GM-CSF was able to regulate cell differentiation and, at the same time, apoptosis in SaOS-2 cells, employing IGF-I as a negative control, and okadaic acid as a positive control of apoptosis.

Some morphofunctional aspects of bone cell cultures suggestive of a *more mature* state confirm that GM-CSF acts as a differentiative rather than a proliferative cytokine on these cells, as happens in other cell cultures. For example, the production of mineralized ECM seems to be promoted by GM-CSF, as revealed by a time-dependent increase in Ca^{++} precipitates in stimulated SaOS-2 cells, with a more significant effect being observed at high GM-CSF concentrations (100 and 200 ng/mL) and after 14 days of culture. The increase in calcium precipitate deposition is one of the most specific and accepted markers of mineralized matrix production as a consequence of osteoblastic differentiation (Pockwinse et al., 1992). Thus, it is interesting to note the expression pattern of osteopontin, which is

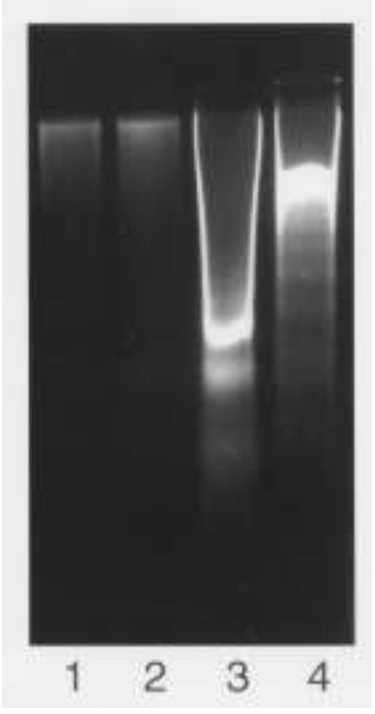

Figure 4, right column. Internucleosomal DNA cleavage of SaOS-2 cells after 14 days of culture. Genomic DNA was isolated and analysed by agarose gel electrophoresis. Unstimulated cells (lane 1); + IGF-I (5 nM) (lane 2); + Ookadaic acid (30 nM) (lane 3); + GM-CSF (200 ng/mL) (lane 4). GM-CSF and okadaic acid generated internucleosomal cleavage that formed a ladder DNA fragment, as is characteristic in cells undergoing apoptosis, while only minimal DNA degradation products were detected in unstimulated or in IGF-I treated SaOS-2 cells.

typical of mature bone tissue, and was known, until just a few years ago, only because it was expressed by osteoclasts in correspondence with the clear zone of the cell membrane, where cells adhere to the bone matrix and exert their remodelling action (Kasugai et al, 1992). Osteopontin is currently considered to be a morphogenetic protein and is widely studied in many tissues, from heart (Graf et al, 1997) to mineralizing bone. The expression of osteopontin became evident very early on in some SaOS-2 cells treated with high concentrations of GM-CSF. However, while in the early days of culture this protein appeared both in the cytoplasm and on the membrane, it was, after a few days, expressed mainly on the cell membrane (Figure 2A). At the same time, BSP-II became lightly evident at 14 days of culture, with its production being increased in GM-CSF treated cells.

Since a known characteristic of osteopontin and BSP-II is that they appear during the last stages of osteoblastic differentiation (Kasugai et al., 1992), the demonstration that SaOS-2 cells produced these two non-collagenous hydroxyapatite crystal-binding proteins at a late stage of culture (14 days), and that their production was increased under GM-CSF stimulation, confirms that GM-CSF triggered SaOS-2 cell differentiation toward a typically *more mature* osteoblastic phenotype.

The synthesis of collagen I, the major specific component of bone connective tissue produced by osteoblasts during differentiation, also seems to be positively modulated by GM-CSF, increasing between 7 and 14 days of culture at 100 and 200 ng/mL. In fact, here we showed that GM-CSF-treated SaOS-2 cells produced collagen I from the early days of culture. The synthesis was initially intracytoplasmatic (data not shown) and then the fibrils appeared organized around the cells and became more evident.

All these data suggest that GM-CSF is able to induce cell differentiation of SaOS-2 cells. Since in the majority of cell systems, the increase in differentiation is associated with the beginning of apoptotic processes (Robbins et al., 1997), we decided to investigate if the same concentrations of the cytokine (200 ng/mL) able to trigger cell differentiation, were also able to induce apoptosis of SaOS-2 cells. It is evident that GM-CSF did not affect SaOS-2 apoptosis after 1 and 7 days of culture, as determined by flow cytometric analysis, whereas it significantly induced it after 14 days of culture, increasing both annexin V incorporation and propidium iodide staining in stimulated cells, thus doubling the percentage of apoptotic cells. This observation coincides with the major effect exerted by the cytokine on synthesis and differentiation. On the contrary, okadaic acid elicited a time-dependent increase in cell apoptosis, so confirming its function as an apoptotic inducing agent in SaOS-2 cells.

Extrinsic and intrinsic apoptotic pathways inducing cells to initiate and execute the cell death process have been identified (Green, 2000). The critical regulators of the intrinsic pathway are the Bcl-2 family members (Vander Heiden et al. 1999), among which Bcl-2 and Bcl-XL are the anti-apoptotic members (Gottlieb, 2000). Thus, to explore the mechanisms that might contribute to the GM-CSF induction of apoptosis, and to determine if they are related to down-regulation of the Bcl-2 protein, we measured its levels by flow cytometric analysis. The results showed that the expression of Bcl-2, either in the stimulated or unstimulated cells, did not change during the culture period. This suggests that GM-CSF stimulated apoptosis in SaOS-2 cells was not associated with a down-regulation of this protein, and presumably was not mediated by this protein.

According to Duke et al. (1983), the analysis of endogenous endonuclease activity by agarose gel electrophoresis of DNA fragmentation shows the formation of a 200bp DNA ladder. This result is consistent with the activation of endogenous endonuclease which cleaves at interucleosomal sites and confirms that SaOS-2 treated cells were undergoing classical apoptosis.

On the other hand, we found that GM-CSF inhibited DNA synthesis in human SaOS-2 osteosarcoma cells after 14 days of culture, results which are consistent with the hypothesis that GM-CSF blocks SaOS-2 cell transit through the G1 phase of the cell cycle. Here we showed that GM-CSF was able to trigger cell differentiation through *in vitro* mineralization and induce BSP-II, osteopontin and collagen I production. At the same time, GM-CSF is a cytokine that activates apoptosis of SaOS-2 cells in vitro after 14 days of culture at a concentration of 200 ng/mL. This apoptotic pathway is not associated with a down-regulation of Bcl-2 protein.

Our results suggest that GM-CSF stimulated a more mature osteoblastic phenotype in SaOS-2 cells and may be involved in the subsequent activation of cellular pro-apoptotic pathways. Further studies on the GM-CSF-induced apoptosis and its mechanism

in the SaOS-2 cell line are required since they may shed new light on the biological aspects of this intriguing phenomenon. On the other hand, the identification of the signal transduction pathways involved in GM-CSF-induced apoptosis of SaOS-2 cells should open the way to a better understanding of the physiological control of bone cells *in vivo*.

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