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# Low Doses and High Doses of Heparin Have Different Effects on Osteoblast-Like Saos-2 Cells In Vitro

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**Abstract** Long-term treatment with heparin has been associated with an increased risk of osteoporosis. Given the importance of heparan sulfate proteoglycans for bone metabolism, it can be anticipated that heparin due to its structural similarity with heparan sulfate chains somehow interferes with the biological activities of these cell surface- and extracellular matrix-associated molecules. Initially in order to study the effect(s) of heparin on osteoblasts that possibly contribute to the development of heparin-induced osteoporosis, we treated osteoblast-like Saos-2 cells in monolayer culture for different periods of time with different concentrations of heparin. None of the heparin concentrations tested led to an inhibition of osteoblast proliferation during the early proliferative phase. After longer incubation times, however, cultures treated with higher concentrations of heparin ($\geq$5 μg/ml) exhibited a reduction in cell number as well as an inhibition of matrix deposition and mineralization. These effects could not be observed with lower heparin concentrations. On the contrary, low concentrations of heparin (5–500 ng/ml) even promoted matrix deposition and its subsequent mineralization. Apparently, heparin has a biphasic effect on osteoblast-like Saos-2 cells, being inhibitory at high concentrations but stimulatory at low concentrations. These results imply that heparin at concentrations well below those used for antithrombotic therapy might eventually turn out to be beneficial for bone formation. J. Cell. Biochem. 91: 1062–1073, 2004. 

**Key words:** Saos-2 cells; osteoblasts; heparin; proliferation; mineralization; apoptosis

Bone formation during both development and remodeling is regulated by hormones as well as by locally acting growth factors that together control proliferation and differentiation of osteoprogenitor cells. Most of the growth factors involved in the regulation of cells of the osteoblast lineage, for example, FGFs, TGF-β1, BMP-2 and -4, IGF-II, are heparin-binding growth factors, so called due to their ability to interact with heparin [Bernfield et al., 1999; Canalis, 2000]. Under physiological conditions, however, the ligand for these growth factors is not heparin, but the structurally related heparan sulfate chains. Heparan sulfate proteoglycans are synthesized by most, if not all nucleated mammalian cells, and are targeted to the cell membrane or to the extracellular matrix. Apparently, these proteoglycans are of great importance for bone. Most of the known derangements of heparan sulfate proteoglycan metabolism exhibit, among others, a pronounced skeletal phenotype. Prominent examples are deletions of the glypican-3 gene, which in humans are the cause of Simpson-Golabi-Behmel syndrome [Pilia et al., 1996; Cano-Gauci et al., 1999], and loss-of-function mutations of the glycosyl transferases that are required for the polymerization of heparan sulfate chains, causing hereditary multiple exostoses [Lind et al., 1998; McCormick et al., 1998]. As mentioned above, the importance of heparan sulfate proteoglycans for bone is inherent to their ability to bind via their heparan sulfate chains most of the growth factors involved in the regulation of bone cell metabolism. These interactions modulate the biological activities of growth factors by a number of ways, for example, by protecting them against proteolytic degradation, by contributing to the formation of growth factor

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gradients and to the storage of growth factors in the extracellular matrix, and by facilitating the interaction of growth factors with their signaling receptors [Bernfield et al., 1999; Lindahl, 1999]. From this, it can be anticipated that alterations of the normal functions of heparan sulfate proteoglycans, either due to derangements of biosynthesis or degradation, or due to interference by exogenous substances, will have profound physiological consequences.

Heparin, which is not synthesized by osteoblasts and osteoclasts but only by connective-tissue type mast cells, is in wide clinical use to prevent and treat thromboembolic disease. Due to a shared biosynthetic pathway, heparin is structurally related to heparan sulfate chains [Lindahl, 2000; Rabenstein, 2002]. It is therefore not surprising that heparin interferes with the ligand-binding activities of heparan sulfate chains by competing for binding sites on "heparin-binding" proteins, thus interfering with normal growth factor responses of cells [Tessler et al., 1994; Larnkjaer et al., 1995; Lindahl, 1999]. Of interest in this context is the association of an increased risk of osteoporosis with long-term heparin-treatment [Griffith et al., 1965; Miller and DeWolfe, 1966; Nelson-Piercy, 1998]. Though little is known about the molecular mechanisms leading to reduced bone mass during long-term heparin treatment, it is reasonable to hypothesize that interference with growth factor signaling in bone cells leading to reduced bone formation by osteoblasts and/or to increased bone resorption by osteoclasts at least contributes to the pathogenesis of heparin-induced osteoporosis.

To assess the effect(s) of heparin on osteoblasts, we treated osteoblast-like Saos-2 cells for different periods of time with different concentrations of heparin. Interestingly, we observed a biphasic response of Saos-2 cells to heparin. Whereas higher concentrations of heparin (5 μg/ml and more) inhibited matrix deposition and mineralization and led to a decrease in cell number, low doses of heparin even promoted matrix deposition and mineralization. The biphasic nature of the effect of heparin on osteoblast-like cells may explain some conflicting reports of heparin effects on osteoblasts. Moreover, the stimulatory effects of low-dose heparin raise the important question of whether these effects could be exploited clinically for stimulating bone formation, for example, in the treatment of osteoporosis.

## MATERIALS AND METHODS

### Materials

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), and trypsin/EDTA solution were purchased from Biochrom (Berlin, Germany). Ascorbic acid 2-phosphate, β-glycerophosphate, heparin from porcine intestinal mucosa (180 USP U/mg), alizarin Red-S and SIGMA FAST p-nitrophenyl phosphate were from Sigma (Taufkirchen, Germany). Thiazolyl blue tetrazolium bromide was purchased from Fluka (Taufkirchen, Germany). RNeasy Mini Kit, RNase-free DNase, Omniscript reverse transcriptase and HotStarTaq DNA polymerase were from Qiagen (Hilden, Germany), the pCR4-TOPO cloning vector was from Invitrogen (Karlsruhe, Germany), and all primers were synthesized by MWG Biotech (Munich, Germany) except for the random hexanucleotide primers which were from Roche Diagnostics (Mannheim, Germany). All other chemicals used were of analytical grade.

The human osteosarcoma cell line Saos-2 was from the American Type Culture Collection (Rockville, MD).

### Cell Culture

Cells were stored in liquid nitrogen in 5% (v/v) dimethyl sulphoxide in DMEM containing 10% FCS. Cells were initially plated into 75-$cm^2$ culture flasks (approximately $1.5 \times 10^4$ cells/$cm^2$) in DMEM containing 44 mM $NaHCO_3$, 2 mM L-glutamine, and 10% FCS (growth medium) and were maintained at 37°C in 10% $CO_2$ in air. Cells were subcultured after reaching approximately 80% confluency by rinsing the cell layer with 0.05% (w/v) trypsin and 0.53 mM EDTA and subsequent incubation at 37°C for 10 min. For experiments, cells were plated at an initial density of $1.0 \times 10^4$ cells/$cm^2$ and maintained in growth medium for 24 h. Then fresh growth medium containing additionally 0.2 mM ascorbic acid 2-phosphate was added and replaced twice a week. For mineralization studies, cultures were further supplemented with β-glycerophosphate at a final concentration of 10 mM (unless otherwise stated) during the last 24 h.

### Cell Proliferation

In the proliferation studies, viable cells were determined by the MTT assay that relies on the ability of mitochondrial dehydrogenases to oxidize thiazolyl blue tetrazolium bromide

(MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to an insoluble blue formazan product. Cells were incubated with heparin for the time periods indicated, washed, and then incubated with growth medium containing 0.3 μM MTT at 37°C for 1 h. After removing the culture supernatant and washing the cells, isopropanol containing 40 mM HCl was added and the optical density of the solution was read at 530 nm in an enzyme-linked immunosorbent assay (ELISA) plate reader.

In some experiments, cell numbers were additionally determined directly by counting the cells after typsinization in a Casy 1 cell counter (Schärfe System, Reutlingen, Germany).

### Matrix Mineralization

For quantification of matrix mineralization, cell cultures were stained with alizarin Red-S essentially as described [Stanford et al., 1995]. Cells were washed with PBS at room temperature and fixed with 4% formaldehyde in PBS. Fixed cell cultures were stained with 40 mM alizarin Red-S (pH 4.2) for 10 min using an orbital shaker. Due to the solubility of amorphic mineral deposits at pH 4.2 staining under these acidic conditions is considered to be specific for hydroxyapatite crystals. To remove non-specifically bound stain, cultures were washed five times with deionized water and once with PBS for 15 min at ambient temperature. Bound dye was solubilized in 10 mM sodium phosphate (pH 7.0) containing 10% cetylpyridinium chloride and quantitated spectrophotometrically at 562 nm. Quantitation by this method has a degree of accuracy similar to quantitation of hydroxyapatite by binding assay [Schiller et al., 1999].

### Staining Procedures

Collagenous matrix was stained by a modified trichrome staining procedure using hematoxylin, Orange G, phosphomolybdic acid, and Anilin Blue water soluble (HOPA) as described [Blahser and Muschke, 1978]. Collagenous matrix is stained dark blue by this procedure.

In addition to the alizarin Red-S staining procedure used for quantification, mineralization was examined histochemically by von Kossa staining. Briefly, fixed cultures were treated with 5% $AgNO_3$ and 1% pyrogallol and the stain was fixed with 5% $Na_2SO_3$. Nuclei were counterstained with Nuclear Fast Red (0.01% in 75 mM aluminium sulfate).

### Determination of Alkaline Phosphatase Activity

Enzymatic activity of alkaline phosphatase, an early marker of osteogenic differentiation, was determined after culturing the cells for 7 days in the presence of the indicated heparin concentrations using SIGMA FAST p-nitrophenyl phosphate as substrate according to the instructions of the manufacturer. In short, cells (10-$cm^2$ growth area) were washed with PBS and lysed in 300 μl 20 mM Tris (pH 10) containing 0.1% (v/v) Triton X-100, 0.5 mM $MgCl_2$, and 0.1 mM $ZnCl_2$. Immediately before measurement, 50 μl of the cell lysate was mixed with 800 μl of substrate solution. Substrate hydrolysis was determined by reading the absorption at 405 nm over a time period of 4 min, and enzyme activities were calculated from the initial reaction velocities.

### RT-PCR

Total RNA was extracted from the cells cultured in the presence of the indicated concentrations of heparin using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and digested with RNase-free DNase (Qiagen) to remove any contaminating genomic DNA. cDNA synthesis from total RNA was performed with Omniscript reverse transcriptase (Qiagen) using $(dT)_{15}$ (1 μM) and random hexanucleotide primers (5 μM; Roche Diagnostics, Mannheim, Germany) simultaneously. Aliquots of the cDNAs were incubated with HotStarTaq DNA polymerase (Qiagen) and the following primers (each at a final concentration of 1 pM): for osteocalcin (accession X04143) 5′-GGC AGC GAG GTA GTG AAG AG-3′ and 5′-AGC AGA GCG ACA CCC TAG AC-3′ (product size 194 bp) and for GAPDH (accession NM_002046) 5′-GAG TCC ACT GGC GTC TTC AC-3′ and 5′-GGT GCT AAG CAG TTG GTG GT-3′ (product size 188 bp). Primer pairs were designed to encompass at least one intron in the genomic sequence to allow for discrimination of any sequences amplified from contaminating genomic DNA. All primers were synthesized by MWG Biotech (Munich, Germany) and were of HPSF-quality. The polymerase was activated (15 min at 96°C) and then 32 cycles (for the amplification of osteocalcin) and 25 cycles (for GAPDH) (45 s at 94°C, 45 s at 60°C, 45 s at 72°C) were performed on a RoboCycler Gradient 96 (Stratagene, Amsterdam, The Netherlands). All amplification

products had the expected size and were verified by cloning into the pCR4-TOPO vector (Invitrogen, Karlsruhe, Germany) and subsequent sequencing. Amplification products were visualized by agarose gel electrophoresis after staining with ethidium bromide. For an estimation of the relative expression, integrated optical densities of the amplification product bands were determined (ImageMaster, Amersham Biosciences, Freiburg, Germany) and normalized to GAPDH.

### Statistical Analysis

All experiments were repeated at least three times, and similar results were obtained in these independent experiments. Quantitative proliferation and mineralization assays too were performed at least in three independent experiments, each in quadruplicate, and means and standard deviations of typical experiments are shown. Data were analyzed using Student's $t$-test. $P < 0.05$ was considered statistically significant.

## RESULTS

### Saos-2 Cells as a Model to Study Heparin Effects on Osteoblasts

In order to investigate the effects of heparin on human osteoblasts, we decided to use the osteosarcoma cell line Saos-2, as these cells have been shown to produce a collagenous extracellular matrix able to mineralize in the presence of an exogenous phosphate donor [McQuillan et al., 1995], and therefore exhibit the entire differentiation program from proliferation to mineralization. When cultured in monolayer for 18 days, these cells yielded a confluent cell lawn with apparent nodule formation that mineralized upon addition of β-glycerophosphate as phosphate donor (Fig. 1a,d). In contrast, treatment with 50 μg/ml heparin for 17 days (starting at day 1) resulted in cultures with scattered cell aggregates leading to remarkable discontinuous mineralized structures (Fig. 1c,f). No obvious effects could be observed upon treatment with 50 ng/ml heparin, though mineralization appeared to be stronger in these cells compared with the untreated control (Fig. 1b,e).

### Effects of Heparin on the Proliferation of Saos-2 Cells

The effects observed after treatment with 50 μg/ml heparin described in the preceding paragraph raised the question whether high concentrations of heparin inhibit the proliferation of Saos-2 cells. We, therefore, studied the effects of different concentrations of heparin, ranging from 5 ng/ml to 50 μg/ml, on the proliferation of Saos-2 cells. Whereas no differ-

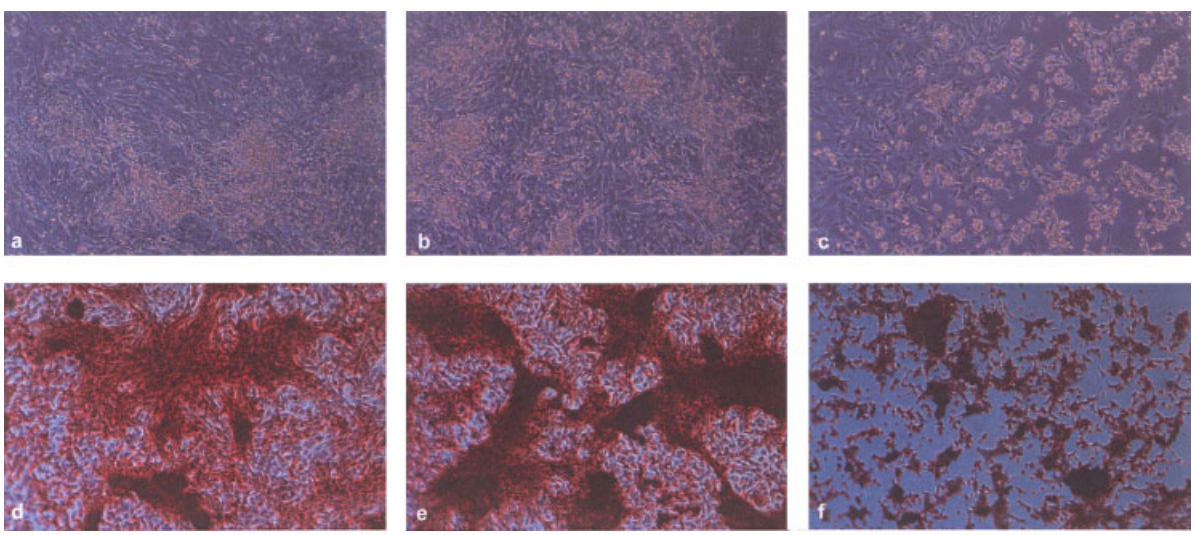


**Fig. 1.** Effect of heparin treatment on Saos-2 cell cultures. Saos-2 cells were seeded at an initial density of 10,000 cell/cm$^2$. After 1 day, the culture medium was replaced by medium containing ascorbic acid 2-phosphate and none (**a**, **d**), 50 ng/ml (**b**, **e**), and 50 μg/ml (**c**, **f**) heparin, respectively. After 16 days, photographs were taken from all cell cultures (a–c) before the addition of 10 mM β-glycerophosphate. Mineralization was assessed 1 day later by von Kossa staining (d–f), nuclei were counterstained with Nuclear Fast Red. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ences could be seen during the first 2 days, there were significantly fewer cells in the cultures treated with 5 and 50 μg/ml heparin after 13 days (Fig. 2). Comparable results were obtained by directly counting the cells (Fig. 2d), indicating that heparin, even at high concentrations and prolonged incubation times, had no effect on mitochondrial dehydrogenase activity. The reduction in cell number could not be observed in cultures treated with 5–500 ng/ml heparin for the same time. Surprisingly, after 3 days, there were significantly ($P < 0.05$) more cells (up to 136% of non-treated control in the experiment shown in Fig. 2b) in the heparin-treated cultures.

### Effects of Heparin on Matrix Deposition

As heparin due to its structural similarity with heparan sulfate chains might have an influence on cell matrix interactions leading to alterations in the organization of the extracellular matrix deposited by the cells, we treated Saos-2 cells with different concentrations of heparin and visualized the collagenous matrix formed after 14 days by trichrome staining. As can be seen in Figure 3, there was a much denser staining matrix in the cultures treated with 5–500 ng/ml heparin, compared with the untreated control and with the cultures treated with higher concentrations of heparin (5 and 50 μg/ml).

### Effects of Heparin on the Mineralization of Saos-2 Cell Cultures

Our initial experiments on heparin effects on Saos-2 cell cultures clearly had demonstrated that mineralization is inhibited by heparin at a high concentration (Fig. 1). To further characterize this inhibitory effect, matrix bound mineral was quantitated by staining with alizarin Red-S and spectrophotometric evaluation of bound dye. Essentially no mineral deposition could be observed without prior incubation with β-glycerophosphate (Fig. 4). After incubation with 5 mM β-glycerophosphate for 24 h, there was strong mineralization in the cultures not treated with heparin. Mineralization in the cultures treated with 5 μg/ml heparin was inhibited by 75%. Increasing the concentration of the phosphate donor led to a slight, though significant ($P < 0.05$) increase (by 9%) of mineral deposition in the cultures not treated with heparin and to a further decrease of mineralization in the heparin-treated cultures by 43% ($P < 0.05$).

In contrast to the inhibitory effect of high concentrations of heparin (5 μg/ml heparin and more), lower heparin concentrations surprisingly turned out to promote the mineralization of Saos-2 cell cultures (Fig. 5). Strongly staining and connected mineral-containing structures were formed when the cell cultures were treated with 50 and 500 ng/ml heparin, respectively (Fig. 5a), and mineral content increased consistently under these conditions ($67 \pm 42\%$ with 50 ng/ml and $161 \pm 63\%$ with 500 ng/ml, respectively, in three independent experiments, each performed in quadruplicate) compared with the heparin-free control.

### Effects of Heparin on Markers of Differentiation

The unexpected effects of low heparin concentrations on matrix deposition and mineralization raised the question whether heparin influences osteogenic differentiation of Saos-2 cells. We, therefore, studied activity of alkaline phosphatase, an early marker of osteogenic differentiation, and expression of osteocalcin, a late differentiation marker. As can be seen from Figure 6, both 5 and 50 ng/ml heparin lead to a significant increase in the activity of alkaline phosphatase after 7 days. This increase can not be accounted for by increased cell numbers, as after 7 days there are no significant differences in cell number between the cultures not treated with heparin and those treated with low concentrations of heparin (<5 μg/ml). After 14 days, a concentration-dependent increase in osteocalcin expression can be observed that is most prominent at the highest heparin concentrations used (Fig. 7).

## DISCUSSION

Given the well-known association of long-term heparin treatment with osteoporosis, our results relating to the inhibitory effects of high heparin concentrations on osteoblast function are not surprising. Indeed, an inhibitory effect of heparin on bone nodule formation by mouse calvaria cells in vitro has already been reported [Bhandari et al., 1998]. In that study, the number of bone nodules formed was reduced by approximately 90% after incubating the cells in the presence of 3.1 μg/ml heparin for 21 days. Using quantitation of total mineral incorporation into the matrix, we observed a similar inhibitory effect for the human osteosarcoma cell

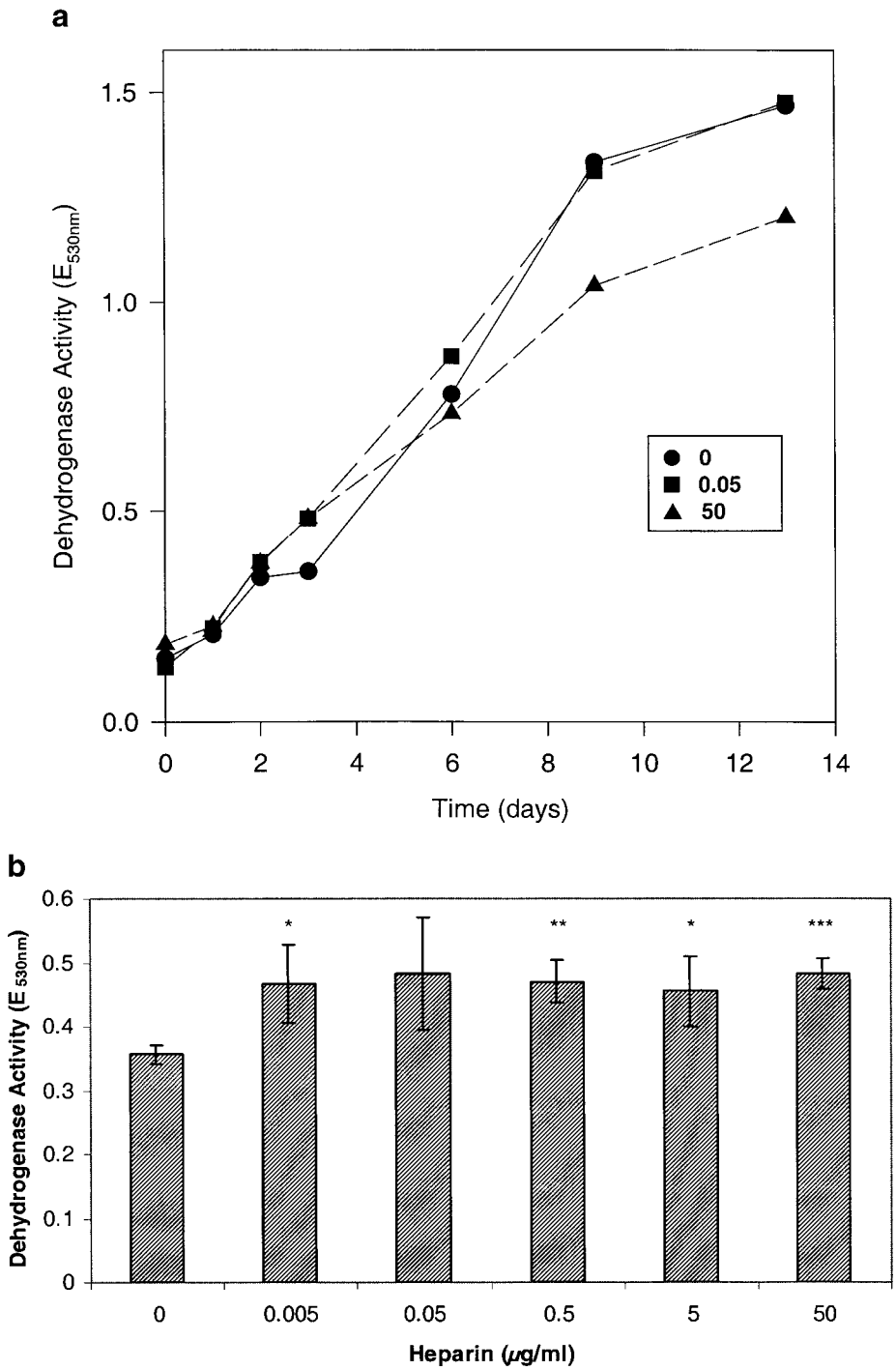


**Fig. 2.** Effect of heparin treatment on proliferation of Saos-2 cells. Saos-2 cells were seeded at an initial density of 10,000 cell/$cm^2$. After 1 day, the culture medium was replaced by medium containing ascorbic acid 2-phosphate and different concentrations of heparin. Cell numbers after different incubation times in the presence of heparin were determined by assessment of mitochondrial dehydrogenase activity (**a–d**) and direct cell counting (d). a: Proliferation of Saos-2 cells in the presence of none (●), 50 ng/ml (■), and 50 μg/ml (▲) heparin, respectively. Mitochondrial dehydrogenase activity after 3 days (b) and 13 days (c) in the presence of the indicated concentrations of heparin. d: Comparison of cell number and mitochondrial dehydrogenase activity after 14 days in the presence of the indicated concentrations of heparin. Results of a typical experiment performed in quadruplicate are shown; values are given as means ± SD (omitted in A for clarity). *Different from control ($P < 0.05$); **different from control ($P < 0.01$); ***different from control ($P < 0.001$).

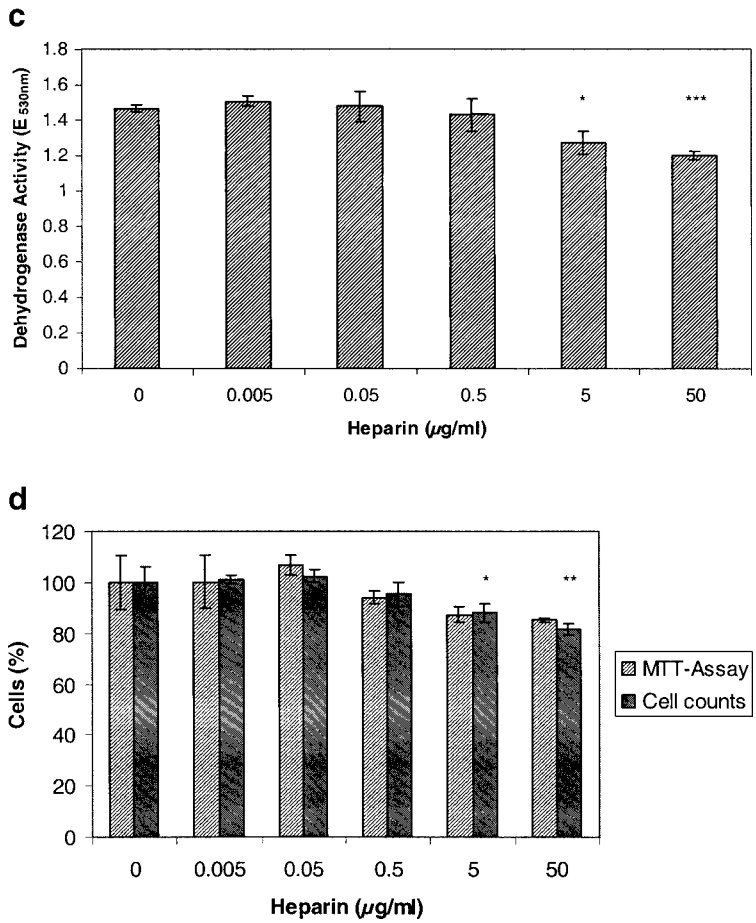


**Fig. 2.** (*Continued*)

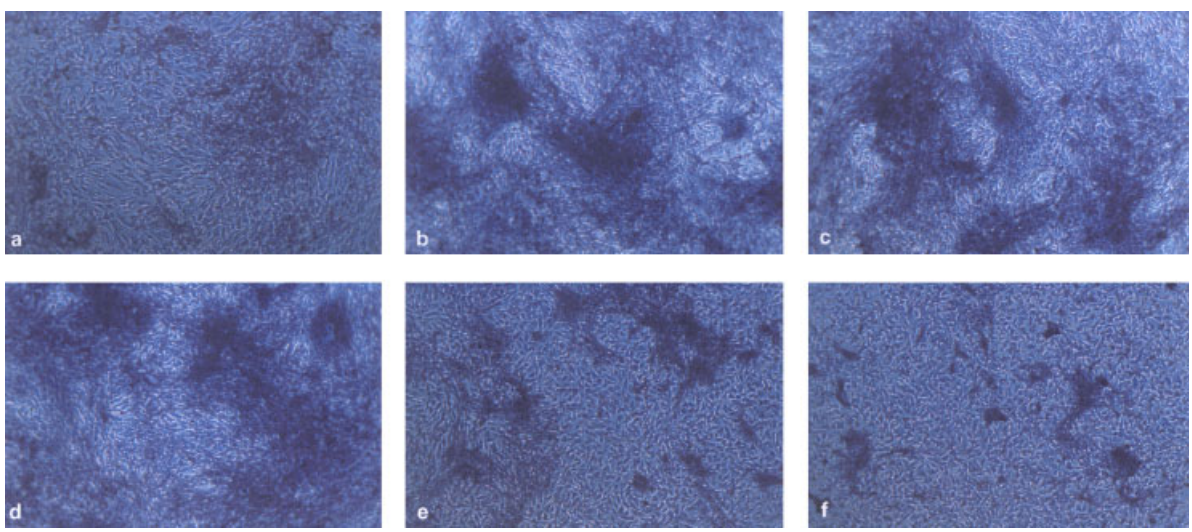


**Fig. 3.** Effect of heparin treatment on matrix deposition in Saos-2 cell cultures. Saos-2 cell cultures were treated for 14 days with none (**a**), 5 ng/ml (**b**), 50 ng/ml (**c**), 0.5 µg/ml (**d**), 5 µg/ml (**e**), and 50 µg/ml (**f**) heparin, respectively. Collagenous matrix was visualized by trichrome staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

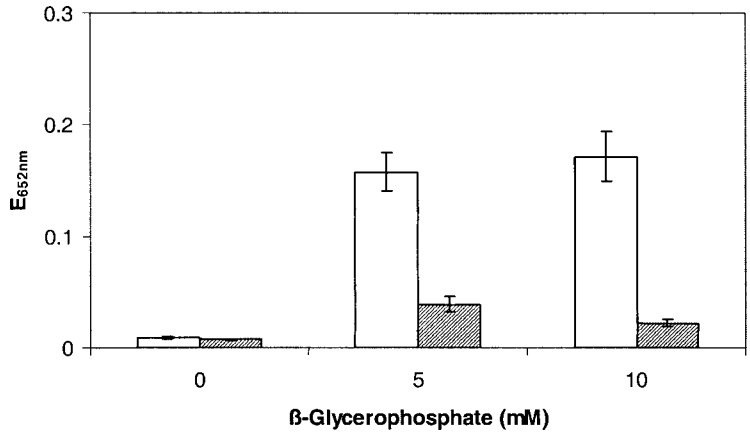


**Fig. 4.** High concentrations of heparin inhibit the mineralization of Saos-2 cell cultures. Saos-2 cells were cultured for 13 days in the absence (open bars) or presence of 5 μg/ml heparin (filled bars) and subsequently for another day in the presence of the indicated concentrations of β-glycerophosphate. Mineralization was assessed by staining with alizarin Red-S under acidic conditions. Bound dye was quantitated photometrically after desorption. Results of a typical experiment performed in quadruplicate are shown; values are given as means ± SD.

line Saos-2 after preincubation with 5 μg/ml heparin, when mineralization was induced by addition of 10 mM β-glycerophosphate.

Reports on the effects of heparin on the proliferation of osteoblastic cells are conflicting. Thus, both inhibitory and promoting effects have been stated recently [Kock and Handschin, 2002; Matziolis et al., 2002]. These conflicting descriptions mostly appear to be related to different experimental conditions, such as the heparin concentrations used and the incubation times employed. Indeed, in both studies, an inhibitory effect of high heparin concentrations was observed. From our results presented here, it becomes apparent that a negative influence of high concentrations of heparin becomes evident only after about 1 week, whereas the growth promoting effect

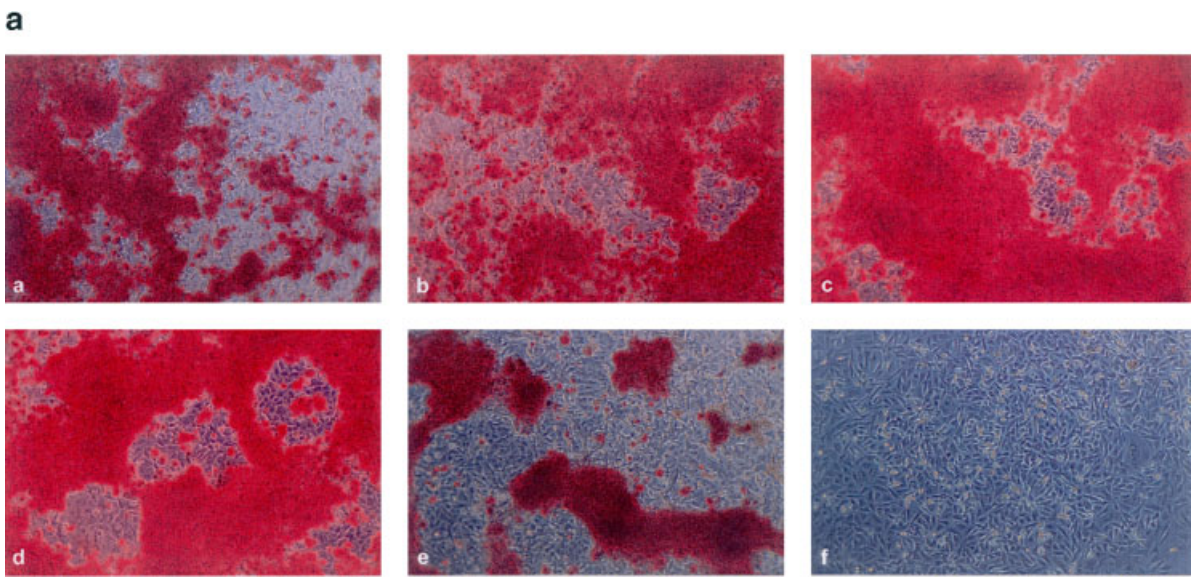


**Fig. 5.** Low concentrations of heparin promote the mineralization of Saos-2 cell cultures. Saos-2 cell cultures were treated for 15 days with the heparin concentrations detailed in Figure 3. Mineralization was visualized by staining with alizarin Red-S (**a**). Bound dye was solubilized and quantitated photometrically (**b**). Results of a typical experiment performed in quadruplicate are shown; values are given as means ± SD. *Different from control ($P < 0.05$); **different from control ($P < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

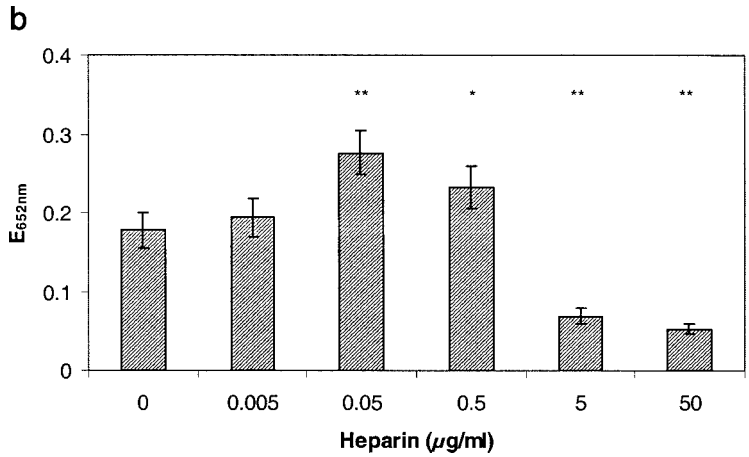


**Fig. 5.** (*Continued*)

of heparin appears to be limited to the initial proliferative phase. This growth promoting effect of all heparin concentrations tested argues against an inhibitory effect of heparin on proliferation per se. However, reduced cell numbers after prolonged incubation times are not necessarily the result of reduced proliferation rate, but could also be attributed to reduced cell survival. It is tempting to speculate that high concentrations of heparin might render osteoblast-like cells more sensitive to apoptosis, possibly by interfering with the transmission of survival signals from extracellular heparin-binding growth factors. In line with this, preliminary data indicate that prolonged incubation with high concentrations of heparin-lead to a decrease in the bcl-2/bax ratio (unpublished observation). Such a mechanism could well contribute to the pathogenesis of heparin-induced osteoporosis by diminishing the osteoblast population, thus leading to an imbalance of bone resorption and bone formation. Accordingly, a decrease in osteoblast surface has been described in heparin-treated rats [Muir et al., 1996]. It should be kept in mind, however, that in vivo heparin not only influences osteoblasts, and effects on osteoclasts have to be taken into account [Walton et al., 2002].

The most unexpected finding of the present study was the demonstration of a promoting effect of low heparin concentrations (5–500 ng/ml) on matrix deposition and mineralization. This stimulatory effect could not be attributed to an increased cell number, as at the time points these effects were observed there were no significant differences in the cell numbers of

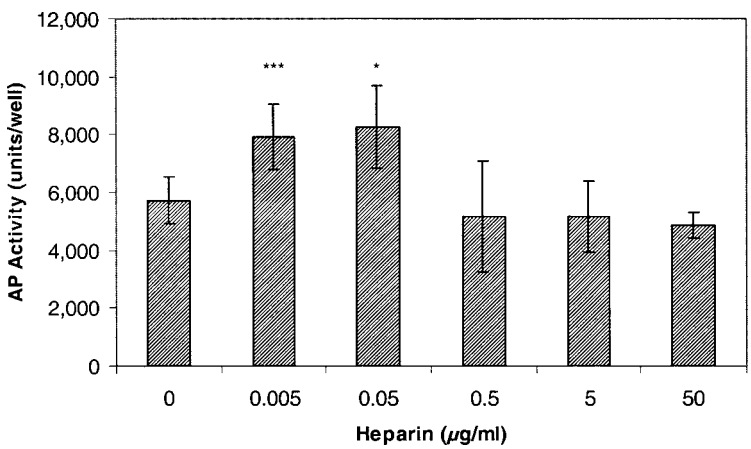


**Fig. 6.** Effect of heparin on the activity of alkaline phosphatase. Saos-2 cell cultures were treated for 7 days with the heparin concentrations indicated. Enzymatic activity of alkaline phosphatase was assayed using p-nitrophenyl phosphate as substrate. Results of a typical experiment performed in quadruplicate are shown; values are given as means ± SD.

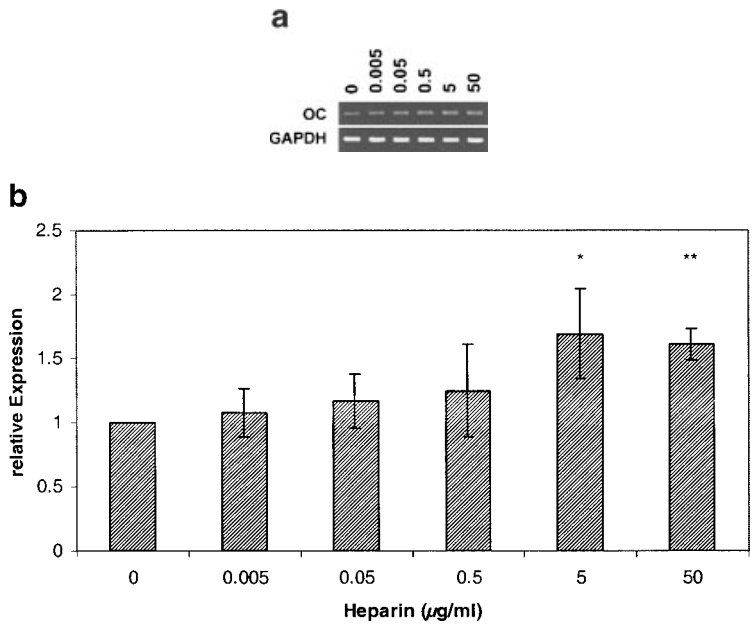


**Fig. 7.** Effect of heparin on the expression of osteocalcin. Saos-2 cell cultures were treated for 2 weeks with the heparin concentrations indicated. Osteocalcin expression was assayed by means of reverse transcriptase polymerase chain reaction. **a**: Amplification products were analyzed by agarose gel electrophoresis. **b**: An estimate of the relative expression was made by determining the integrated optical densities of the amplification product bands. Results of three independent experiments are shown; values are given as means ± SD. *Different from control ($P < 0.05$); **different from control ($P < 0.01$).

the cultures not treated with heparin and the cultures treated with low heparin concentrations. Instead, heparin apparently had an influence on the osteogenic differentiation of the Saos-2 cell cultures, as after 1 week the activity of alkaline phosphatase was increased in the cultures treated with 50 and 500 ng/ml heparin, and after 2 weeks a dose-dependent increase of osteocalcin expression could be observed in the heparin-treated cultures. Previous reports on heparin effects on collagen biosynthesis have been conflicting. Whereas, Shibata et al. [1992] found collagen expression to be increased in mineralized MC3T3-E1 cell cultures, Hurley et al. [1992] reported an inhibitory effect of heparin on collagen synthesis in fetal rat calvaria cells. To the best of our knowledge, no data on a mineralization promoting effect of heparin have been published so far. For treatment of pregnant women with thromboembolism, heparin doses up to 40,000 IU/day are used [Dahlman et al., 1994], resulting in plasma levels of about 0.5 IU/ml as judged by antifactor Xa assay [Levine et al., 1994]. This corresponds to a heparin concentration in plasma of approximately 2–3 μg/ml. Though no data are available with respect to the local concentrations of heparin at the sites of active osteoblasts, Shaughnessy et al. [1999] have demonstrated that heparin becomes accumulated in bone tissue during long-term treatment, suggesting that during antithrombotic therapy the heparin concentrations faced by the osteoblasts are well above those we have found to be stimulatory for matrix mineralization in vitro. It appears, therefore, possible that a low-dose heparin regimen might turn out to be beneficial for bone formation in vivo, too. In line with this, dextran-derived heparan-like polymers able to interact with heparin-binding growth factors have recently been reported to promote bone healing in rat craniotomy defects [Colombier et al., 1999]. This undoubtly deserves closer examination.

At present, only speculations can be made how heparin exerts its biphasic effect on osteoblast-like cells. However, heparin-binding growth factors that are involved in the regulation of osteoblast metabolism and survival appear to be an attractive target. As most of the growth factors involved in the regulation of osteoblasts are heparin-binding, the observed effects will most probably be attributable to heparin influences on more than one signaling pathway. A particularly interesting target for heparin interference in osteoblasts, however, might be the bone promoting members of the

Wnt protein family. Signaling by Wnt-proteins has been shown to be dependent on heparan sulfate proteoglycans in *Drosophila* [Baeg and Perrimon, 2000; Selva and Perrimon, 2001], and it is most likely to be in humans, too. Signaling in bone is dependent on the presence of LDL receptor-related protein 5 (LRP5), and mutations in this protein have recently been shown to be responsible for specific forms of high-bone-mass trait and osteopenia, respectively [Boyden et al., 2002; Kato et al., 2002; Little et al., 2002]. Biphasic effects are not uncommon for heparin [Chevreuil et al., 1993; Keil et al., 1995; LaRochelle et al., 1999]. Regarding a single ligand-receptor system, low concentrations of heparin may facilitate the encounter of the growth factor with its signaling receptor by a "reduced dimensionality" mechanism [Lander, 1999], thus promoting the growth factor effect, whereas higher concentrations may be inhibitory due to a saturation of heparin-binding sites. Obviously, the situation becomes even more complex when multiple receptor-ligand systems with different affinities and signaling properties are involved. Here the same concentration of heparin may be stimulatory on one pathway and simultaneously be inhibitory on another pathway due to different ligand affinities. Further studies are required to elucidate the contribution of heparan sulfate proteoglycans to the regulation of osteoblastic cells and to understand the interference of these processes by heparin.

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