Systemic effects of severe trauma on the function and apoptosis of human skeletal cells

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Systemic factors are believed to be pivotal for the development of heterotopic ossification in severely-injured patients. In this study, cell cultures of putative target cells (human fibroblastic cells, osteoblastic cells (MG-63), and bone-marrow stromal cells (hBM)) were incubated with serum from ten consecutive polytraumatised patients taken from post-traumatic day 1 to day 21 and with serum from 12 healthy control subjects.

The serum from the polytraumatised patients significantly stimulated the proliferation of fibroblasts, MG-63 and of hBM cells. The activity of alkaline phosphatase in MG-63 and hBM cells was significantly decreased when exposed to the serum of the severely-injured patient. After three weeks in 3D cell cultures, matrix production and osteogenic gene expression of hBM cells were equal in the patient and control groups. However, the serum from the polytraumatised patients significantly decreased apoptosis of hBM cells compared with the control serum (4.3% vs 19.1%, p = 0.031).

Increased proliferation of osteoblastic cells and reduced apoptosis of osteoprogenitors may be responsible for increased osteogenesis in severely-injured patients.

Heterotopic ossification (HO) is characterised by the formation of normal bone around bones or joints at ectopic sites which are not contiguous with the normal skeleton, leading to complete ankylosis in 10% to 16% of cases. After severe head injury, the incidence is between 10% and 40%. Garland and O’Halloran observed HO in 90% of patients with a combination of head injury and blunt trauma to the elbow. In addition, enhanced healing of fractures and the formation of hypertrophic callus have been reported in severely-injured patients. Thus, a systemically-increased osteogenic potential must be assumed in these patients. The first clinical symptoms of HO are swelling, pain, warmth and erythema. They occur after three to four weeks; formation of bone can be detected radiologically at four to six weeks after trauma. Therefore, biochemical alterations and cellular events leading to HO must evolve within the first three weeks after trauma.

Various aetiologies have been suggested for the development of HO such as a genetic predisposition based on human leukocyte antigens (HLA-B18 and HLA-B27), local changes of expression of bone-morphogenetic protein as present in fibrodyplasia ossificans progressiva and immobilisation or vigorous mobilisation of joints. However, because HO occasionally occurs at uninjured, remote, regions, systemic factors are believed to be pivotal for the induction of HO. Of such factors, basic fibroblast growth factor (bFGF) was found to be elevated in patients with a head injury. Furthermore, factors involved in repairing nerve tissue such as nerve growth factors may also be involved. Chalmers et al postulated that in addition to an inducing factor, two conditions must be met for the formation of bone in soft tissues, namely, the presence of osteogenic precursor cells and an environment which is permissive to osteogenesis. A mechanically-traumatised soft tissue may represent such an ‘ideal’ environment, since HO develops most often at the site of a fracture or in bruised tissue. The concomitant haematoma may deliver the osteogenic precursor cells necessary to synthesise bone matrix.

Our study tests the hypothesis that serum from severely-injured patients affects the proliferation, osteogenic differentiation and apoptosis of osteogenic precursors or mature skeletal cells.

Patients and Methods
Ten consecutive polytraumatised patients (3 women, 7 men with a mean age of 35 years (22 to 45)) were treated at our level-1 trauma centre according to the Advanced Trauma Life Support protocol.
Support guidelines.\textsuperscript{20} After primary diagnostic and therapeu-
tic management, they were transferred to the inten-
se care unit. All gave informed consent and the study had eth-
ical approval.

The criteria for polytrauma were met if the injury sever-
ity score (ISS) was more than 16 points and a systemic
trauma reaction, e.g. systemic inflammatory response syn-
drome, was present.\textsuperscript{21-23} The latter was assumed if a
patient had two or more of the following clinical findings:
temperature $> 38.8^\circ$ or $< 36.0^\circ$, heart rate $> 90$ beats/
minute, respiration rate $> 20$/minute or peripheral arterial
carbon dioxide ($\text{PaCO}_2$) $< 32$ mmHg, leucocytosis
$> 12$ 000/mm$^3$ or leucopenia $< 4000$/mm$^3$. The mean pre-
operative ISS was 42 points (17 to 57). Seven of the ten
patients had sustained a severe head injury which was dem-
onstrated by haemorrhagic lesions or diffuse cerebral
eodema on cerebral CT scans. Of these, three needed a
craniotomy and two required monitoring of the intra-
cranial pressure. Blood samples were collected in the inten-
sive care unit at days 1, 3, 5, 7 and 10 from a central
venous line, and at days 14 and 21 from a peripheral vein.
They were immediately centrifuged and the resulting serum
was stored at $-70^\circ$C.

In the post-traumatic period, two patients developed
pneumonia and one showed positive bacterial cultures
from the cerebrospinal fluid after two craniotomies. All
septic complications were successfully treated with anti-
biotics. One patient developed a severe acute respiratory
distress syndrome, but recovered completely.

The patients were followed clinically for a mean of 17
months (12 to 25) after their injury. Three patients who had
a head injury developed HO within 12 weeks which had to
be resected because of limited movement of the affected
joint.

Control blood samples were obtained from 12 healthy
subjects (eight women, four men) with a mean age of 38
years (21 to 65). Preliminary data showed that the effects of
serum did not depend on gender or cigarette smoking.

**Cells.** A human fibroblastic cell line (HS-27), a human
osteoblastic cell line (MG-63), and human bone-marrow
stromal cells (hBM) were used for the bioassays. Femoral
bone marrow was obtained as discarded material from a
79-year-old woman (subject A) undergoing total hip
replacement and from a 22-year-old man (subject B) after
abundant bone grafting. Low-density mononuclear cells
were isolated by density centrifugation on Ficoll-Histo-
paque 1077 (Sigma; Gibco/BRL Laboratory, Grand Island,
New York). Mononuclear cells were cultured in 10% fetal
bovine serum. Therefore, 10% human serum was used in all experiments.

**Two-dimensional (monolayer) assays.** Two thousand cells
(HS-27, MG-63 and hBM from both subjects) were seeded
in 96-well plates in seven replicates and were incubated with
10% human serum from the ten patients at each time-point
(post-traumatic day 1 to day 21) and from the 12 control
subjects. Cell proliferation was assessed by a colorimetric
assay (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetra-
solium bromide) as described by Mosmann.\textsuperscript{24} After five
days, 10 $\mu$l of MTT solution were added. After incubation
for four hours at 37$^\circ$C, 100 $\mu$l of 0.04 N HCl in isopropanol
were added to each well. The amount of formazan cleaved
by living cells was measured as absorbance at 570 nm with
a microplate reader. Activity of alkaline phosphatase was
assessed after five days using the $p$-nitrophenol method. The
measurements were corrected to the amount of cells esti-
mated by the MTT assay after five days. The results were
expressed as ratios. For both sets of experiments the values
were expressed as the mean $\pm$ SEM of the ten patients and 12
control subjects with seven replicates each.

**Three-dimensional cell culture.** Three-dimensional collagen
sponges were made, as previously reported.\textsuperscript{25,26} Briefly, a
solution of 0.5% pepsin-digested collagen from bovine skin
(Cellagen; ICN Biomedical, Costa Mesa, California) was
neutralised with 4-(2-hydroxyethyl)-1-piperazineethane
sulfonic acid (HEPES) and NaHCO$_3$ and 250 $\mu$m of this
collagen solution were poured into a mould and frozen at
-20$^\circ$C. After lyophilisation, each side of the collagen sponge
was irradiated by ultraviolet light for three hours.

Three hundred thousand hBM (from subject B) were
seeded onto two replicates of ten collagen sponges. Each
replicate was cultured with 10% human serum from poly-
traumatised patients with HO (three sponges), from poly-
traumatised patients without HO (three sponges) and from
control subjects (three sponges). One sponge was cultured
in 10% fetal bovine serum. In order to mimic the situation
in vivo, serum from trauma patients was introduced into
the cell cultures in a chronological order over three weeks,
i.e. serum from post-traumatic day 3 was given at day 3 of
cell culture and so forth.

**Histological analysis.** After three weeks in culture, one set
of ten sponges was collected for histological analysis and
was fixed in paraffin. Sections were stained with 0.5% tolui-
dine blue O (Fisher Scientific, Pittsburgh, Pennsylvania)
and with von Kossa\textsuperscript{27} for semiquantitative estimation of
matrix production and mineralisation. Other sections were
processed for TUNEL assay (terminal transferase dUTP nick
end labelling, \textit{in situ} cell death detection kit; Boe-
hringer, Mannheim, Germany) to detect DNA fragmenta-
tion. The number of TUNEL-positive cells and the total
number of cells were counted at 400 x magnification using
a grid eyepiece which defined an area of 0.04 mm$^2$. The
percentage of positive cells was determined for three ran-
domly-selected fields in triplicate sections from a total of
three samples per treatment group (control subjects and
patients) and was statistically analysed using the unpaired
Student’s \textit{t}-test.
Table I. The Primer sequences

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<th>Primer*</th>
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*GADPH, glyceraldehyde-3-phosphate dehydrogenase; Col 1, collagen type 1; Bcl-2, B-cell leukaemia/lymphoma 2 protein; BAX, Bcl-2 associated X protein

† $T_a$, annealing temperature
‡ bp, base pair

Bar charts showing the effect of serum from the 12 control subjects (C) and ten polytraumatised patients (day 1 to day 21 after trauma) on the number (± SEM) of a) human fibroblastic, b) human osteoblastic and c) human bone-marrow stromal cells.
Reverse transcription-polymerase chain reaction (RT-PCR) analysis. After three weeks, another set of ten collagen sponges was collected for molecular analysis. Messenger-RNA was extracted with TRI-zol reagent (Invitrogen Life Technologies, Carlsbad, California) following the manufacturer’s instructions and 2 µg of total RNA was reverse-transcribed into cDNA with SuperScript II (Invitrogen Life Technologies). One-tenth of the cDNA was used in 50 µl PCR reactions (Table I) with gene-specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen type I, alkaline phosphatase, and osteocalcin as previously reported.28,29 Primers for the anti-apoptotic Bcl-2 (B-cell leukaemia/lymphoma 2 protein) and for the pro-apoptotic BAX (Bcl-2 associated X protein) were designed from the gene nucleotide bank (Table I). The intensity of the PCR bands for gene transcripts was ranked in a masked manner from grade 0 to 5 and was expressed for each gene transcript in relation to the intensity of GAPDH as a housekeeping gene.

Enzyme-linked immunosorbant assay (ELISA). Serum levels of putative growth factors (basic fibroblast growth factor (bFGF)), β-nerve growth factor (β-NGF) were measured from day one to day 21 using human immunoassays following the manufacturer’s instructions (QuantiKine; R&D systems, Minneapolis, Minnesota). The lower and upper detection limits for these ELISA assays were 3 pg/ml to 1000 pg/ml for bFGF and 8 pg/ml to 500 pg/ml for β-NGF.

Statistical analysis. For the two-dimensional experiments the values were expressed as the mean ± SEM of ten patients and 12 control subjects with seven replicates each. Statistical analysis of the normally distributed data was carried out by one-way analysis of variance (ANOVA). A p-value of less than 0.05 was considered to be significant. For assessment of the ratio between the estimated mRNA expression of Bcl-2 and of BAX and fraction of apoptotic cells, logarithmic regression analysis was used. Statistical analyses were performed using the StatView software (version 4.51; Abacus Concepts Inc., Berkeley, California).

Results

Proliferation and alkaline phosphatase activity. Serum from the polytraumatised patients taken on days 7, 14 and 21 showed a significantly increased number of HS-27 (134% of control, p < 0.001) and MG-63 cells (160% of control, p < 0.001; Figs 1a and 1b), whereas the number of hBM cells decreased with serum from day 1 to 5 (74% of control at day 1, p < 0.05) and increased with serum from day 14 to day 21 (132%, p < 0.05; Fig. 1c). Serum from the polytraumatised patients taken in the first ten post-traumatic days significantly decreased the alkaline phosphatase activity of osteoblasts (32% of control, p < 0.01) and of hBM cells (56% of control) (p < 0.05, Fig. 2) compared with serum from control subjects. Serum from the patients who developed HO did not differentially affect cell proliferation or alkaline phosphatase activity compared with that from patients without HO.

Histological analysis. After three weeks, hBM cells cultured in 3D collagen sponges showed good viability, accumulated extracellular matrix and were positive for von Kossa staining. There were no differences in matrix accumulation or calcification between sponges cultured in serum from control subjects and from polytraumatised patients. However, the sponges treated with serum from the control subjects showed significantly more apoptotic cells (19.1% SD 11.2) than those treated with serum from polytraumatised patients (4.3%, SD 5.0, p = 0.031). The effect of 10% fetal...
bovine serum was equal to that of the control subjects (21.5% apoptotic cells). The anti-apoptotic effect was even more pronounced when hBM cells were cultured in the serum from those polytraumatised patients who developed HO (1.2% SD 0.3, p = 0.012, Fig. 3).

**RT-PCR analysis.** Human hBM cells cultured in 3D collagen sponges for three weeks showed expression of mRNA or bone-specific markers such as collagen type 1, osteocalcin and alkaline phosphatase (Fig. 4). There were no differences between cells cultured in serum from polytraumatised patients and those from control subjects. However, as estimated by semi-quantitative RT-PCR analyses, mRNA expression for Bcl-2 was elevated in hBM cells cultured in serum from polytraumatised patients (estimated intensity of PCR bands relative to GAPDH: 2/5, 3/5, 4/5, 5/5, 0/5, 2/5, Fig. 4) compared with hBM cells cultured in control serum (estimated intensity of PCR bands relative to GAPDH 3/5, 0/5, 3/5, 5/5, 0/5, 2/5; Fig. 4) and increased with serum from control subjects (estimated intensity of bands relative to GAPDH: 3/5, 2/5, 1/5). The ratio between the estimated mRNA expression of Bcl-2 and of BAX significantly correlated with the fraction of apoptotic cells (R = 0.463, p = 0.0149) estimated by the TUNEL assay.

**ELISA.** Serum levels of bFGF from polytraumatised patients were increased fivefold (27.5 pg/ml ± SEM 17.6 at day 7, p < 0.05) compared with control serum (5.2 pg/ml ± SEM 3.7, Fig. 5). The levels from patients with clinically apparent HO did not differ from those of the polytraumatised patients who did not develop HO. Serum levels of β-NGF were not increased after trauma.

**Discussion**

Serum from polytraumatised patients affected the proliferation, function and apoptosis of fibroblastic and osteoblastic cells. Proliferation of HS-27, MG-63 and in part of hBM cells was significantly increased with a peak stimulatory effect at day 10, whereas the alkaline phosphatase activity of MG-63 and of hBM cells was significantly reduced with serum from the first ten days. This observation is consistent with the biological principle that cells either divide or produce extracellular matrix at any one time. The increased proliferation rate of HS-27, MG-63 and to a lesser extent of hBM cells can be explained by the
Mean ± SEM serum levels of basic fibroblast growth factor (bFGF) from the 12 control subjects (C) and from ten polytraumatised patients (day 1 to day 21).

Mean ± SEM serum bFGF (pg/ml)

Days

Fig. 5

elevated levels of bFGF found in the blood samples from polytraumatised patients. In line with this observation, the peak stimulatory effect and the highest level of bFGF were both at day 10. Surprisingly, serum from patients with HO did not differentially affect skeletal cells in the 2D experiments. It is conceivable that this is because not all patients had the concomitant soft-tissue injury necessary to develop clinically apparent HO. Therefore patients with and without HO may have equally increased osteogenic potentials.

The data from our study are partially in contrast to those of earlier reports. Bidner et al. found that serum from patients with a head injury stimulated proliferation of rat calvaria osteoblastic cells, but not of fibroblastic cells. Klein et al. obtained serum from rats at various time intervals after exposure to a severe head injury. Serum taken at days one and two after trauma significantly decreased the number of bone-marrow stromal cells but increased alkaline phosphatase activity. The discrepancies between these studies and our study may be because of the different types of cell used and their stage of maturation as well as the time points at which the blood samples were taken. Furthermore, most animal models are of limited value, because post-traumatic HO only occurs in man, the cat and the dog.

In our study, 3D collagen sponges were used for the long-term cultures, because they allow high-density cultivation and provide a geometry similar to that found in vivo. Thus, this culture system avoids cell necrosis and dedifferentiation seen in monolayer long-term cultures. Furthermore, to mimic the in vivo situation, serum from polytraumatised patients was given in chronological order over three weeks. Human hBM cells cultured in 3D sponges showed good viability and accumulated extracellular matrix, but semiquantitative histological analysis revealed no differences in matrix accumulation, cell number or calcification. Likewise, mRNA isolated from hBM cells after three weeks in culture showed expression of markers specific for osteogenic differentiation, but there were no semi-quantitative differences between the treatment groups. However, as detected by TUNEL assay, the number of apoptotic hBM cells was significantly decreased when 3D sponges were cultured with serum from polytraumatised patients compared with control serum and fetal bovine serum. This effect was even more pronounced when cultures were treated with serum from patients who developed HO. Furthermore, the ratio between mRNA expression of anti-apoptotic Bcl-2 and mRNA expression of pro-apoptotic BAX significantly correlated with the fraction of apoptotic cells estimated by the TUNEL assay. This suggests that the anti-apoptotic effect of serum from polytraumatised patients may be mediated by changes of Bcl-2 and BAX expression. However, the mechanism of this anti-apoptotic effect has not been elucidated in our study. Elevated levels of bFGF may not be responsible because bFGF tends to promote apoptosis of osteoblastic cells, β-NGF, which is known to inhibit osteoblast apoptosis, was not elevated in the serum of polytraumatised patients, although it is markedly increased in the cerebrospinal fluid after a head injury. However, for haemopoietic cells, inhibition of apoptosis after severe trauma has been repeatedly described. It has been shown that apoptosis of neutrophil granulocytes is decreased by pro-inflammatory mediators such as interferon-γ, tumour necrosis factor-α, interleukins (IL-1β, IL-6, IL-10) and colony-stimulating factor, which are elevated in the early post-traumatic course. It is conceivable that this mechanism may also pertain for osteoblastic precursor cells, but proof of this hypothesis is beyond the scope of this study.

In conclusion, we have demonstrated that serum from polytraumatised patients stimulates proliferation of fibroblastic, osteoblastic and, in the later post-traumatic course, osteoprogenitor cells. In addition, we have shown histological and molecular evidence that apoptosis of osteoprogenitors is significantly inhibited. These effects may be responsible for enhanced osteogenesis in severely-injured patients.

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References


