Eur J Med Res (2008) 13: 292-298

Tissue Inhibitor of Metalloproteinases II (TIMP-2) is an Osteoanabolic Factor in vitro and in vivo

F.-J. Kramer[1], M. Meyer[2], D. Morgan[3], W.-G. Forssmann[4], L. Ständker[4], H. Schliephake[1], S. Mark[2], E. Maronde[2,5]

[1]Department of Oral and Maxillofacial Surgery, George-August-University of Goettingen, Germany
[2]IPF PharmaCeuticals GmbH, Hanover, Germany
[3]TAP Pharmaceutical Products, Lake Forest, IL, USA
[4]Institute for Pharmacology, Hannover Medical School, Hanover, Germany
[5]Institut für Anatomie III, JW Goethe Universität, Frankfurt. Germany

Abstract

Objective: Critical size defects (CSDs) of bone are defined as defects that do not heal spontaneously to new bone during the lifetime of an adult individual. In contrast, immature animals are capable to heal defects of identical size. It was our hypothesis that age-related paracrine effects are relevant for this difference in regeneration.

Methods: The pooled supernatant of primary rat calvarial osteoblast-like cell cultures (POBC) derived from prenatal or postnatal donors was concentrated and applied into CSDs of adult recipient organisms (n = 10). In addition, the supernatant of POBC derived from prenatal donors was pooled and purified by reverse-phase chromatography. Each pre-purified fraction was tested in a proliferation indicating bioassay. Peptide fractions containing proliferative activities were re-chromatographed and re-tested in a bioassay. Finally, a proliferative activity was purified, identified by sequence analysis and applied into CSDs of adult recipients.

Results: The application of POBC derived from prenatal donors resulted in osseous regeneration of a CSD in adult recipients, while the supernatant of postnatal donors had much smaller effects. The morphologic features resembled the spontaneous osseous healing of calvarial defects of the same size in immature organisms. The polypeptide „tissue inhibitor of metalloproteinases type II"(TIMP-2) was isolated from the supernatant of cultures of POBC derived from prenatal donors by measuring the induction of their proliferation. Additionally, the application of human TIMP-2 injected into calvarial CSDs of adult organisms resulted in osseous healing.

Conclusion: We conclude that one component responsible for the healing effect of CSDs of POBC supernatants derived from prenatal donors is TIMP-2.

Key words: Bone healing, Ageing, Tissue Inhibitor of Metalloproteinases-2 (TIMP-2), Osteoblasts, Critical Size Defect

Abbreviations: POBC: Primary osteoblast-like cell cultures; CSD: Critical size defect

Introduction

It is a well known clinical experience that the regenerative potential of bone is related to the age of an individual [1]. Defects of bone of a certain size that are incapable of spontaneous regeneration by formation of new bone during the lifetime in adult organisms are defined as "critical size defects" (CSD) [2]. Those defects become repaired by a scar-like connective tissue representing a functionally insufficient state of regeneration [3]. Interestingly, immature organisms are competent to heal defects of an identical size completely by the formation of new bone, demonstrating their superior intrinsic potential for osseous regeneration [4].

Similar to these *in vivo* observations, variations of osteoblast biology *in vitro* related to the donor`s age were reported [5]. Primary cultures of calvaria-derived osteoblast-like cells (POBC) were shown to exhibit a reduced proliferation and differentiation capacity when derived from elder donor organisms [6]. It was our hypothesis that paracrine acting substances synthesized by POBC of prenatal donors are responsible for these age-related effects. Therefore, aim of the present study was to characterize the biological potential of POBC supernatants derived from pre- and postnatal donor ages and to identify functionally relevant substances within the supernatants.

Methods and Statistics

Materials

All experiments were approved by the local animal care committee (Bezirksregierung Hannover, Germany).

Primary Cell Cultures

Rat calvarial osteoblasts were prepared as described previously [7, 8]. Donor organisms were prenatal Wistar rats at day 21 of gestation and immature Wistar rats of 21 days of postnatal age. In brief, calvariae were removed and the attached soft tissue was dissect-

ed. The calvarial preparations were cut into small pieces and submersed in PBS. After intense rinsing with PBS the bone pieces were incubated sequentially in a solution containing Collagenase P and Trypsin. The isolated suspension was filtered through a 0.2 mm metal mesh filter and centrifuged at 800 rpm. The pelleted cells were resuspended, counted and seeded at a density of 100.000 cells per 10 cm Petri dish. Media (Minimum essential medium (MEM), containing 10% fetal bovine serum (FBS)) were changed every 2-3 days. Cells were washed the next day and split after 10 days. Cells derived from these preparations were used for the cell culture experiments described here.

For the collection of serum-free supernatants cells were washed once with PBS and incubated (37°C, 5% CO_2 atmosphere) for 24 hours with MEM without FBS. Subsequently, the cells were allowed to regrow in MEM containing MEM for 24 hours. This procedure was repeated up to one week.

In vivo Effects of Prenatal versus Postnatal Supernatant

The CSD model of the calvaria was choosen because of its easy and reliable surgical management and its wide distribution for the analysis of bone healing, testing of bone substitute materials, DNA vectors or peptide growth factors [9-12]. The serum-free culture supernatants were collected and pooled for each age group. Five litres of each supernatant were purified by reverse-phase chromatography and concentrated by batch-elution. After lyophilization, the eluates were reconstituted in sterile 0.9% sodium chloride solution salt solution, transferred onto collagen carriers (Lyostypt®; Braun, Melsungen, Germany) and implanted into fresh critical size defects (8 mm diameter) of the calvaria of adult Wistar rats (n = 10 per group). The control group was implanted with collagen carriers containing 0.9% sodium chloride solution. After 2, 4 and 8 weeks the healing process was analyzed radiographically and histologically (Alizarin red staining).

Isolation of Active Components

Serum-free supernatant from calvarial osteoblasts derived from prenatal donors was collected and pooled. Isolation of the activity was done by stepwise chromatographic separation. After each chromatographic step an activity test in the rat calvarial cell proliferation assay using WST-1 was done. In brief, cells were seeded in 96-well plates at a density of 5,000 cells per well in serum-free medium. Aliquots of the chromatographic fractions were added directly after seeding and the induction of proliferation was measured after two days of incubation. WST-1 detects mitochondrial lactate dehydrogenase activity which reflects cell number and vitality. WST-1 assay-derived values correlated well with cell counting using a hemocytometer, a Coulter counter or a CASY device. The identified active fractions were repeatedly chromatographed and tested until isolation.

For peptide isolation several chromatographic steps were performed: first the supernatant was applied to a preparative YMC column (Gel Basic, 15-30 µm, 47 × 300 mm). Subsequently, the active fractions were applied onto a Biotek RP silica C4 column (10 nm, 5 µm, 20x125 mm). The active fractions obtained were further separated using analytical reverse-phase material (YMC C18, 12 nm, 5 µm, 4.6 × 250mm). Finally, a Phenomenex C4 column was used (30 nm, 5 µm, 4.6 × 250 mm). The partial sequence of the isolated factor was determined by N-terminal sequencing and the molecular mass by MALDI-MS as described [13, 14].

Application of TIMP-2

Experiments were performed with TIMP-2 purified from rat prenatal POBC or human MG-63 cells [15] and with commercially available recombinant human TIMP-2 (Triple Point Biologicals, Portland, Oregon, USA) with identical results. 1 µg (low dose) or 10 µg (high dose) of TIMP-2 were dissolved in 0.9% sodium chloride solution and applied in a volume of 150µl into collagen carriers (Lyostypt®; Braun, Melsungen, Germany). The loaded carriers were implanted into fresh CSD (8 mm diameter) of the calvaria of adult Wistar rats (n = 10 per group). In a third group (n = 10) 2.5 µg of bone morphogenic protein-2 (BMP-2), an osteoinductive peptide (R&D Systems, Minneapolis, USA), was implanted into the CSD. Finally, in a control group (n = 10) collagen carriers containing 0.9% sodium chloride solution only were implanted. Additionally, at the first, second and third day after surgery identical dosages of TIMP-2 and BMP-2 as applied intraoperatively were dissolved in 150µl of 0.9% sodium chloride solution and injected locally into the collagen carriers implanted in the calvarial defects. In the control group 150µl of 0.9% sodium chloride solution were injected. After an observation period of 2, 4 and 8 weeks the healing process was analyzed radiographically and histologically (Alizarin red staining). The tissue density of the regenerates was analysed by computer tomography. The tissue density scores were collected in a data base and compared by Student's t-test. The data are presented as means and ±SD. Values of $p \leq 0.05$ were considered as statistically significant.

Results

Radiographic analysis of bone healing (Fig. 1) showed no spontaneous formation of new bone in the control animals 2, 4 or 8 weeks after surgery (Fig.1 A-C). Likewise, preparations from POBC derived from postnatal donors did not induce relevant bone formation in CSD healing of adult rats (Fig. 1D-F). Only small amounts of new bone formation could be detected 8 weeks after the implantation. This new bone was exclusively located at the margins of the defect. In the centre of the defect no bone formation was observed. On the contrary, treatment with the supernatant derived from prenatal donors resulted in a strong induction of bone formation after only 4 weeks and was increased even stronger after 8 weeks (Fig. 1 G-I). Interestingly, in this group the formation of new bone was located predominantly in the centre of the defect.

Histological analysis (Fig. 2) of bone healing confirmed the results obtained from radiography. Staining

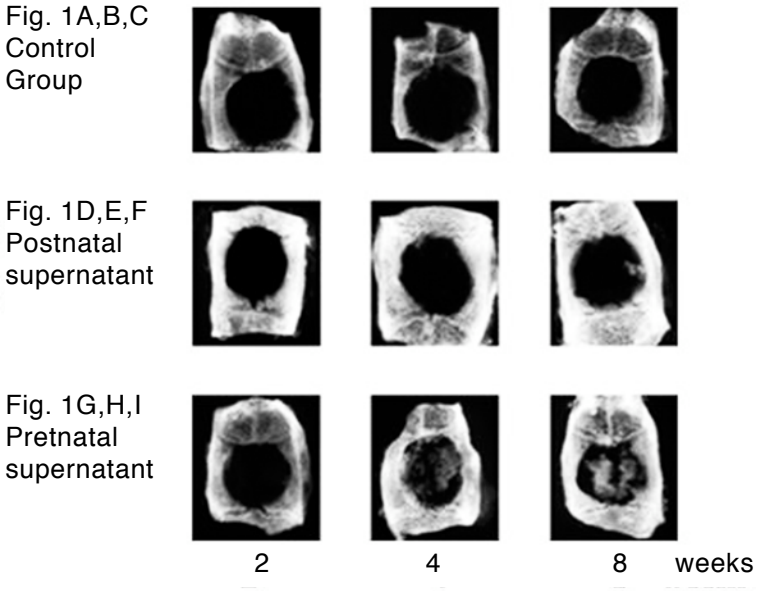


Fig. 1A-I. Radiographs of critical size defect healing with/without application of POBC supernatant: Radiographs were taken after 2,4 and 8 weeks of healing in critical size defects of adult rat calvaria after application of purified and concentrated supernatants derived from POBC. Donors of POBC were either prenatal or postnatal rat. While in the control group (Fig. 1 A-C) and in the group with postnatal supernatant (Fig. 1 D-F) no relevant bone formation was observed defects treated with supernatant of POBC of prenatal donors showed pronounced new bone formation after 4 and 8 weeks (Fig. 1 G,H,I).

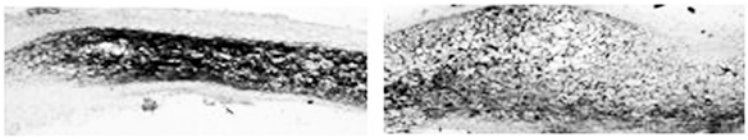


Fig. 2A

Fig. 2D


Fig. 2B

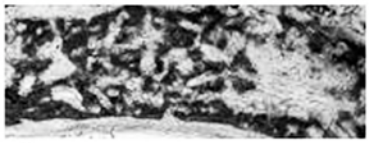


Fig. 2E

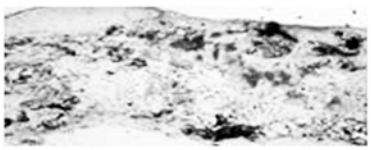


Fig. 2C

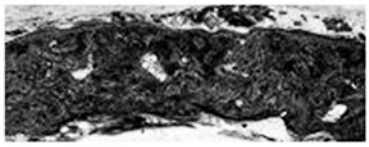


Fig. 2F

Fig. 2. Histological analysis of CSD healing after application of POBC supernatants: Fig. 2 A, B, C: Application of supernatants derived from cultures of postnatal donors (Alizarin red; 12x). The observation period extended 2 weeks (Fig. 2 A), 4 weeks (Fig. 2 B) and 8 weeks (Fig. 2 C). Only small islands of bone formation were found after 8 weeks (Fig. 2 C). Figure 2 D,E,F: Application of supernatants from cultures of prenatal donors. The observation period extended 2 weeks (Fig. 2 D), 4 weeks (Fig. 2 E) and 8 weeks (Fig. 2 F). Formation of new bone was found at the entire defect centre after 4 weeks (Fig. 2 E), resulting in complete osseous bridging after 8 weeks postoperatively (Fig. 2 F).

with Alizarin Red demonstrated that defects treated with the supernatant of cells derived from postnatal donors did not heal by the formation of new bone (Fig. 2 A-C). After resorption of the collagen carrier, only a few osseous islands embedded in a connective, scar-like tissue could be observed at the end of the experiment (Fig. 2 C). In contrast, application of the supernatant of POBC derived from prenatal rats resulted in a strong bone induction (Fig. 2 D-F). Already 4 weeks after surgery formation of new bone was observed all over the defect (Fig. 2 E), and 8 weeks after surgery a complete osseous bridging of the defect was reached (Fig. 2 F).

We collected five litres of prenatal POBC supernatants, subjected them to chromatographic separation, identified a proliferation-inducing activity and used estimation of proliferation activity to isolate this factor (Fig. 3 A-D). After purification, the isolated compound was characterized (Fig. 4 A,B). MALDI-MS analysis resulted in a molecular mass of 21,719 kDa (Fig. 4 A) and N-terminal (Edman-) sequence analysis in the sequence XSXSPVHPQQAFXNADVVIRAKAVE (Fig. 4 B; X corresponds to amino acids that could not be determined). Thus, the purified protein represented full length TIMP-2. The presence of TIMP-2 and of MMP-2 but not of MMP3, MMP-9 and MMP–13 in POBC-supernatants of prenatal donors was also confirmed by ELISA measurements (data not shown).

To test the hypothesis if TIMP-2 was contributing to healing of the CSD in adult rats, both purified TIMP-2 and commercially available human TIMP-2 (hTIMP-2) were applied in the rat CSD model. Similar to the purified TIMP-2, the hTIMP-2-treated animals showed enhanced bone healing with the higher

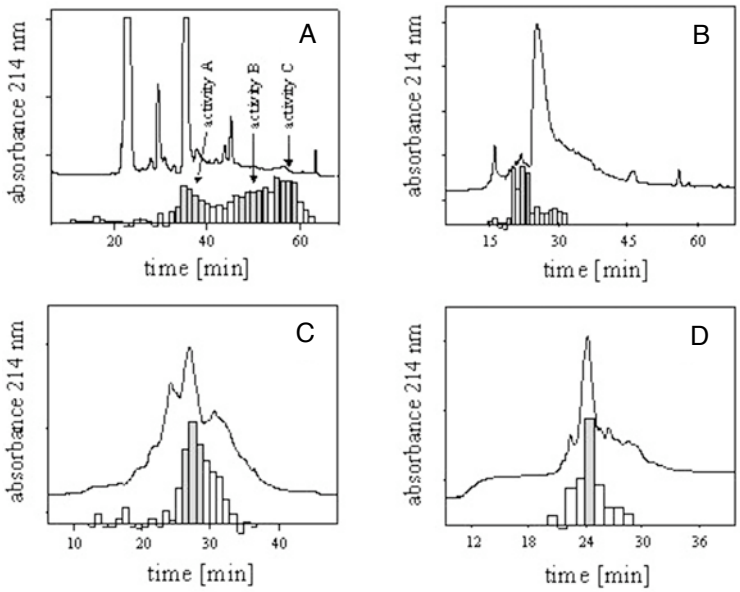
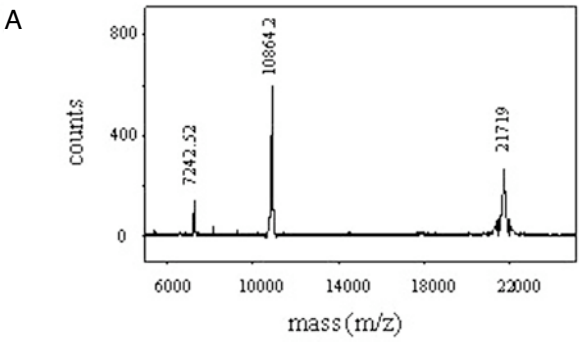


Fig. 3 A-D. Isolation of proliferative activities from supernatants of POBC derived from fetal donors: Fig. 3 A) Activity profile of the peptide fractions generated from the pooled cell culture supernatant. The purification of activity C resulted finally in the isolation of TIMP-2 after several analytical chromatographies (Fig. 3 B,C,D).



B

```
    10         20         30         40         50
----|----|----|----|----|----|----|----|----|----|
CSCSPVHPQQAFCNADVVIRAKAVSEKEVDSGNDIYGNPIKRIQY...
::::::::::::::::::::::::::::
XSXSPVHPQQAFXNADVVIRAKAVSE
```

Fig. 4 A, B. Biochemical analysis of the isolated rat TIMP-2. Determination of the molecular mass by MALDI mass spectrometry (Fig. 4 A). The N-terminal sequence analysis of the purified peptide matched the detected mass and confirmed the isolation of full length TIMP-2 (Fig. 4B).

dose being more effective when compared to control animals (Fig. 5). The positive control BMP-2, an osteoinductive drug, was most effective (Fig. 5). Histological preparations show that in vehicle-treated control animals only fibrous, scar-like tissue was formed (Fig. 5 A) whereas under both hTIMP-2 concentrations (Fig. 5 B, C) and BMP-2 (Fig. 5 D) new mineralized bone was formed. TIMP-2 related formation of bone was always found to occur directly by intramembranous (direct) ossification (Fig. 5 B, C). The BMP-2 treated defects seemed more advanced in bone healing since signs for ongoing remodeling processes are more evident under BMP-2 treatment than under hTIMP-2 treatment (Fig. 5 D). The tissue density of the regenerates was higher after TIMP-2 application (low) ($p<0.022$) when compared to the control group. Application of BMP-2 resulted in a higher tissue density than TIMP-2 application (high) ($p<0.034$) (Fig. 5 E).

Discussion

Bone healing in a postnatal organism is a specific biological process that recapitulates several aspects of prenatal skeletogenesis [16, 17]. Both, postnatal bone healing and prenatal skeletogenesis are processes that include the recruitment, proliferation and differentiation of cells capable to develop a mature osteoblastic phenotype, resulting in bone formation [18]. However, the potential of postnatal bone healing is related to the age of an individual, suggesting an intrinsic difference between immature and adult individuals [6]. It might be hypothesized that some of those differences might be overcome if paracrine acting signalling substances involved in prenatal skeletogenesis are used to support postnatal bone healing. Therefore, primary cell cultures of rodent calvaria derived from prenatal donors were established and shown to develop a mature osteoblast phenotype [7]. It was

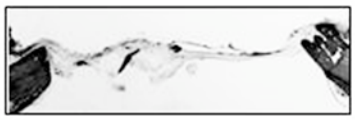


Fig. 5A

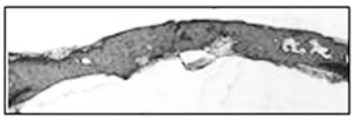


Fig. 5C

Fig. 5B

Fig. 5D

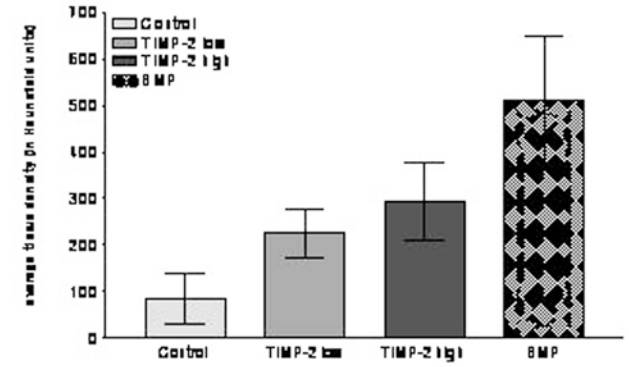


Fig. 5E

Fig. 5 A-E. CSD healing after application of hTIMP-2: In the control group no bone formation was found (Fig. 5 A). Application of hTIMP-2 resulted in formation of bone during CSD healing (Fig. 5 B). The amount and density of bone was enhanced when the concentration of hTIMP-2 was increased (Fig. 5 C). The amount of bone in defects that were treated with BMP-2 was highest (Fig. 5 D). The effects of hTIMP-2 on tissue density were quantified by computer tomography after CSD healing for 8 weeks (Fig. 5 E; n=10; mean + STD). Control defects were treated with 0.9% sodium chloride solution or with BMP-2. hTIMP-2 "low " represents a dose of 1μg per application; hTIMP-2 "high" represents a dose of 10 μg per application. Tissue density was higher after TIMP-2 application (low) ($p<0.022$) when compared to a control group. BMP-2-application resulted in a higher tissue density than TIMP-2 application (high) ($p<0.034$) (Fig. 5 E).

the aim of the current study to evaluate if osteoblastic synthesis/secretion products found in cultures of prenatal donors possess the capability to support bone healing in otherwise non-healing defects of adult individuals.

Application of pooled concentrated prenatal osteoblast supernatant resulted in an improvement of bone healing, leading to complete osseous bridging of an otherwise non-healing defect in adult organisms within only 8 weeks. In contrast, supernatants taken from postnatal donors exhibited a much smaller osteogenic effect. The data presented here imply the existence of certain osteoblast synthesis products found in the supernatant of prenatal donors that are capable to maintain bone formation also in postnatal life. The imitation of a molecular environment similar to that found during prenatal skeletogenesis was shown to support also postnatal bone healing.

Interestingly, the effects of the supernatant depended on the age of the donor organism, suggesting that the signalling molecules relevant for bone formation *in vivo* are synthesized only up to a certain stage of development. Applying a series of peptide purification procedures and bioassays addressing (pre-)osteoblast proliferation in a cell line capable to develop a differentiated osteoblast phenotype [19], one of the activities we identified in supernatants of prenatal donors was the "tissue inhibitor of matrix metalloproteinases type II" (TIMP-2).

We also show that application of human TIMP-2 into calvarial CSDs significantly enhanced bone formation in comparison to untreated animals. However, its activity was somewhat less pronounced when compared to bone morphogenic protein-2 (BMP-2), an already approved morphogenic substance applied to support bone healing.

The mechanism of action of TIMP-2 for the observed bone healing effect is not yet clear. The main mode of action previously described for TIMP-2 is the name-giving inhibition of matrix-metalloproteinases (MMPs) [20]. Those are known to be involved in extracellular matrix proteolysis, by which the pericellular environment is modulated [21, 22]. This involves the turnover of the extracellular matrix substances and also the regulation of cell membrane proteins such as growth factors and their receptors [23]. Bone formation *in vivo* is a complex process that can be enhanced by presence of active growth factors and morphogens, enhancing both proliferation and differentiation of osteoblasts and their precursor cells [24]. Consequently, it might be assumed that one mode of action of TIMP-2 is an inhibition of the inhibitory, proteolytic activity of MMPs, probably resulting in locally increased concentrations of certain growth factors relevant for bone formation.

It has also been described that TIMP-2 raises proliferation activity, cAMP-levels and RAS-activity in human osteosarcoma cells (MG-63) [25, 26]. However, raising cAMP-levels leads in some cells to an inhibition of proliferation and in others to enhanced proliferation [27]. Intrestingly, we observed no changes in cAMP- or phospho-MAPK levels in our cell preparations after TIMP-2 application (data not shown). However, when increasing doses of recombinant TIMP-2 are applied onto rat calvarial osteoblastic cell preparations *in vitro* a dose-dependent increase in the level of osteopontin occurred (data not shown). Osteopontin, a matrix protein of bone, has been shown

to recruit "bone-acetylated glycoprotein" (BAG-75) upon secretion, which serves in the extracellular space of osteoblasts as a scaffold for apatite nucleation [28, 29]. Consequently, TIMP-2 may contribute to this process, by stabilizing or even increasing the amount of osteopontin.

Recently another mechanism of action for TIMP-2 has been described in the context of proliferation inhibition in human vascular endothelial cells [30]. It was shown that TIMP-2 binds to and activates a $\alpha 3\beta 1$-integrin receptor which activates a protein tyrosine phosphatase, presumably SHP-1. The proliferation inducing action of TIMP-2 on prenatal rat calvarial cells contrasts to the observed inhibition of proliferation in human vascular endothelial cells and would contradict a role for this mechanism in the intramembraneous new bone formation observed here. However, it is well possible that TIMP-2 induces proliferation in POBCs and inhibits proliferation in other cells. It is even conceivable that an initial inhibition of vascular endothelial proliferation contributes to the bone inducing effect of TIMP-2 application *in vivo*. In conclusion, which mechanisms are responsible for the effects on proliferation *in vitro* and for the formation of new bone *in vivo* is unclear and will be investigated in the future.

In summary we have isolated and identified TIMP-2 as a polypeptide factor capable of supporting intramembraneous bone formation in otherwise non-regenerating defects in adult organisms *in vivo*.

Acknowledgements: We thank for the skillful technical assistance of Ute Block and Daniela Zoch. The authors have no conflicting financial interests.

REFERENCES

1. Chan GK, Duque G. Age-related bone loss: old bone, new facts. Gerontology 2002; 48:62-71.
2. Schmitz JP , Hollinger JO.The critical size defect as an experimental model for craniomandibulofacial nonunions. Clin Orthop Relat Res 1986; 205:299-308.
3. Schmitz JP, Schwartz Z, Hollinger JO, Boyan BD. Characterization of rat calvarial nonunion defects. Acta Anat 4.Einhorn TA. Clinically applied models of bone regeneration in tissue engineering research. Clin Orthop Relat Res 1990; 367:59-67.
5. Erdmann J, Kogler C, Diel I, Ziegler R, Pfeilschifter J. Age-associated changes in the stimulatory effect of transforming growth factor beta on human osteogenic colony formation. Mech Ageing Dev 1999; 110:73-85.
6. Cowan CM, Quarto N, Warren SM, Salim A, Longaker MT. Age-related changes in the biomolecular mechanisms of calvarial osteoblast biology affect fibroblast growth factor-2 signalling and osteogenesis. J Biol Chem 2003; 278: 32005-32013.
7. Bellows CG, Aubin JE, Heersche JNM, Antonsz ME. Mineralized Bone Nodules formed in vitro from enzymatically released rat calvaria cell populations. Calcified Tiss Int 1986; 38:134-154.
8. Aronow MA, Gerstenfeld LC, Owen TA, Tassinari MS, Stein GS, Lian JB. Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvaria cells. J Cell Physiol 1990; 143:213-221.
9. Clockie CM, Moghadam H, Jackson MT, Sandor GK. Closure of critical sized defects with allogenic and alloplastic bone substitutes. J Craniofac Surg 2002;13:111-121.
10. Blum JS, Barry MA, Mikos AG, Jansen JA. In vivo evaluation of gene therapy vectors in ex vivo-derived marrow stroma cells for bone regeneration in a rat critical size calvarial defect model. Hum Gene Ther 2003; 10:1689-1701.
11. Chang SC, Chuang H, Chen YR, Yang LC, Chen JK, Mardini S, Chung HY, Lu YL, Mak WC, Lou J. Cranial repair using BMP-2 gene engineered bone marrow stromal cells. J Surg Res 2004; 119:85-91.
12. Le Guehennec L, Goyenvalle E, Aguado E, Houchmand-Cuny M, Enkel B, Pilet P, Daclusi G, Layrolle P. Small-animal models for testing macroporous ceramic bone substitutes. J Biomed Mater Res 2005; 72:69-78.
13. Lammerich HP, Busmann A, Kutzleb C, Wendland M, Seiler P, Berger C, Eickelmann P, Meyer M, Forssmann WG, Maronde E. Identification and functional characterization of hemorphins VV-H-7 and LVV-H-7 as low-affinity agonists for the orphan bombesin receptor subtype 3. Br J Pharmacol 2003; 138:1431-1440.
14. Fricke K, Schulz A, John H, Forssmann WG, Maronde E. Isolation and characterization of a novel proopiomelanocortin-derived peptide from hemofiltrate of chronic renal failure patients. Endocrinology 2005; 146:2060-2068.
15. Johansen JS, Williamson MK, Rice JS , Price PA. Identification of proteins secreted by human osteoblastic cells in culture. J Bone Miner Res 1992; 7:501-512.
16. Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. J Cell Biochem 2003; 88:873-884.
17. Tuan RS. Biology of developmental and regenerative skeletogenesis. Clin Orthop Relat Res 2004; 427:S105-17.
18. Vortkamp A, Pathi S, Peretti GM, Caruso EM, Zaleske DJ, Tabin CJ. Recapitulation of signals regulating embryonic bone formation during postnatal growth and in fracture repair. Mech Dev 1998; 71:65-76.
19. Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria J Cell Biol 1983; 96:191-198.
20. Stetler-Stevenson WG, Krutzsch HC, Liotta LA. Tissue inhibitor of metalloproteinases (TIMP-2) A new member of the metalloproteinase inhibitor family. J Biol Chem 1989; 264:17374-17378.
21. Lehmann W, Edgar CM, Wang K, Cho TJ, Barnes GL, Kakar S, Graves DT, Rueger JM, Gerstenfeld LC, Einhorn TA. Tumor necrosis factor alpha (TNF-alpha) coordinately regulates the expression of specific matrix metalloproteinases (MMPS) and angiogenic factors during fracture healing. Bone 2005; 36:300-310.
22. Zeitler P, Pahnke J, Marx A. Expression of stromelysin-1 (MMP-3), gelatinase B (MMP-9) and plasminogen activator system during fetal calvarial development. Histopathology 2004; 44:360-366.
23. Murphy G, Knauper V, Lee MH, Amour A, Worley JR, Hutton M, Atkinson S, Rapti M, Williamson R. Role of TIMPs (tissue inhibitors of metalloproteinases) in pericellular proteolysis: the specifity is in detail. Biochem Soc Symp 2003; 70:65-80.
24. Schliephake H. Bone growth factors in maxillofacial skeletal reconstruction – a review. Int J Oral Maxillofac Surg 2002, 31:469-484.
25. Yamashita K, Suzuki M, Iwata H, Koike T, Hamaguchi M, Shinagawa A, Noguchi T, Hayakawa T. Tyrosine phosphorylation is crucial for growth signaling by tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2). FEBS Lett 1996; 396:103-107.
26. Wang T, Yamashita K, Iwata K, Hayakawa T. Both tissue inhibitors of metalloproteinases-1 (TIMP-1) and TIMP-2 activate Ras but through different pathways. Biochem Biophys Res Commun 2002; 296:201-205.

27. Stork PJ , Schmitt JM. Crosstalk between cAMP and MAP kinase signalling in the regulation of cell proliferation. Trends Cell Biol 2002; 12:258-266.
28. Midura RJ, Wang A, Lovitch D, Law D, Powell K, Gorski JP. Bone acidic glycoprotein-75 delineates the extracellular sites of future bone sialoprotein accumulation and apatite nucleation in osteoblastic cultures. J Biol Chem 2004; 279: 25464-25473.
29. Gorski JP, Wang A, Lovitch D, Law D, Powell K, Midura RJ. Extracellular bone acidic glycoprotein-75 defines condensed mesenchyme regions to be mineralized and localizes with bone sialoprotein during intramembranous bone formation. J Biol Chem 2004; 279:25455-25463.
30. Seo D, Li H, Guedez L, Wingfield PT, Diaz T, Salloum R, Wie B, Stetler-Stephenson WG. TIMP-2-mediated Inhibition of Angiogenesis: An MMP-Independent mechanism. Cell 2003; 114:171-180.

Received: January 10, 2008 / Accepted: March 4, 2008

Address fore correspondence:
Priv. Doz. Dr. Dr. Franz-Josef Kramer, MD, DMD, PhD.
Department of Plastic Oral and Maxillofacial Surgery
George-August-University of Goettingen
Robert-Koch-Str. 40
37099 Goettingen
Germany
Phone: +49-(0)551-39-2880
Fax: +49-(0)551-39-2866
E-mail: Franz-Josef.Kramer@med.uni-goettingen.de