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Expression of Colony-Stimulating Factor-1 In Vivo During the Formation of Osteoclasts

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ABSTRACT

Colony-stimulating factor-1 (CSF-1), originally described as a growth factor for macrophages, is essential for the proliferation and differentiation of the cells of the osteoclast lineage. The cytokine is synthesized either as a secreted or a membrane-bound protein, which are encoded by four transcripts. The aim of the present study was to investigate the expression of CSF-1 in vivo at the mRNA level. Transcripts encoding CSF-1 were determined in total RNA from fetal murine metatarsals of different ages by a quantitative reverse-transcription polymerase chain reaction assay. Within the investigated period of time, the bone rudiments contain cells of the osteoclastic lineage representing well-defined differentiation stages. We found that only low levels of transcripts encoding CSF-1 could be detected in metatarsals from 15-day-old fetuses. Transcript levels increased slowly during the following days to reach a maximum in the rudiments from 18-day-old fetuses. After birth, in newborn animals, transcript levels were lowered again. While in rudiments from 15-day-old fetuses a considerable portion of the transcripts encoded the membrane-bound molecule, a transcript encoding the secreted form of the cytokine was the predominant species during the following days. These results suggest that the maintenance of proliferating and postmitotic osteoclast precursors requires low levels of CSF-1 only. Highest levels of locally synthesized CSF-1 are required, however, during the initial recruitment and activation of osteoclasts. After birth, levels of CSF-1 transcripts decrease again, suggesting that newly synthesized CSF-1 may be replaced by protein released from the mineralized matrix during resorption. In conclusion, the present data further strengthen the notion that CSF-1 produced locally acts in a paracrine fashion during the formation of osteoclasts. (J Bone Miner Res 1998;13:1267–1274)

INTRODUCTION

In recent years, it has become evident that colony-stimulating factor-1 (CSF-1), originally described as a growth factor for the cells of the mononuclear phagocytic system, is an essential growth factor for the cells of the osteoclast lineage (for a review see Ref. 1). The elucidation of the role of this cytokine in bone metabolism was greatly facilitated by the demonstration that the murine mutant strain *op* is deficient in CSF-1.[2,3] The phenotype of homozygous *op*/*op* mice is characterized by a reduced number of macrophages and a virtual absence of osteoclasts, the cells resorbing the bone. This lack of osteoclasts causes the deficiency in bone resorption and ultimately the osteopetrosis.[4,5] Upon injections of CSF-1 into *op*/*op* animals, the formation of osteoclasts is restored and the osteopetrotic phenotype is reversed.[6,7] This reversal, however, is not complete. Analysis of the macrophage populations in different organs revealed that some tissue macrophages did not recover to normal levels, despite the treatment with CSF-1.[8] Furthermore, a residual subepiphyseal osteosclerosis persisted in treated animals,[9] suggesting that in the presence of circulating growth factor the recruitment of osteoclasts was still impaired at these sites. Several factors may account for the incomplete recovery: Precursor cells at some sites may not be accessible to circulating CSF-1; different molecular forms of CSF-1 may exert specific, locally restricted effects on their respective target cells that cannot be fully mimicked by the circulating cytokine; and the lack of CSF-1 during development of *op*/*op* animals may cause a deficiency in precursors that are responsive to CSF-1.

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The effects of CSF-1 on the development of osteoclasts led to the investigation of the putative targets for the cytokine and of the cells producing the factor in vivo. These studies revealed that the receptor for CSF-1, which is encoded by the proto-oncogene c-*fms,* is expressed by osteoclast precursors as well as by the mature cells,[10,11] suggesting a direct action of the cytokine on the cells of this lineage. Furthermore, it was demonstrated that cells expressing CSF-1 were located in close proximity to differentiating osteoclast precursors and actively resorbing osteoclasts,[12] supporting the hypothesis that CSF-1 might act in a locally restricted manner during the formation of osteoclasts.

Cell culture models subsequently revealed that CSF-1 was not only required for the formation of osteoclasts,[13] but also for survival of the mature cells.[14] Furthermore, the cytokine was found to inhibit osteoclastic resorptive activity[15] while stimulating migration[16] of the cells. Thus, through its diverse actions on osteoclast precursors and mature osteoclasts, the cytokine might possibly be involved in the regulation of the distribution of resorption sites in bone.

The close proximity of the cells producing CSF-1 and the cells responsive to the growth factor prompted investigations on its expression by osteoblasts. Previously, it was reported that the growth factor is synthesized either as a secreted or a membrane-bound protein.[17,18] Post-translationally, the secreted form is modified by the attachment of a glycosaminoglycan side chain, which may serve as an anchor to incorporate the factor into the extracellular matrix.[19,20] Subsequently, we demonstrated, both on protein and RNA levels, that murine osteoblasts in vitro synthesize the different molecular forms of the cytokine and that these proteins are encoded by four transcripts.[21]

In the present study, the expression of transcripts encoding either the secreted or the membrane-bound forms of CSF-1 was investigated in vivo during the formation of osteoclasts in fetal murine metatarsals by quantitative reverse transcription polymerase chain reaction (qRT-PCR).[22] Fetal metatarsals represent an excellent model to follow the formation of osteoclasts during bone development.[23,24] In the rudiments from 15- and 16-day-old fetuses, locally proliferating, tartrate-resistant acid phosphatase negative osteoclasts precursors are present on the outer diaphyseal surface. One day later, these cells proceed to a postmitotic, tartrate-resistant acid phosphate positive state. At day 18, the precursors fuse, forming mature osteoclasts which invade the rudiment. Subsequently, the bone marrow cavity is eroded and trabecular bone is formed.

Transcripts encoding CSF-1 were found to be expressed at low levels only in metatarsals from 15-day-old fetuses. During the following days, up to a fetal age of 18 days, transcript levels increased steadily. In the metatarsal bones from newborn animals, however, the amount of transcripts encoding the cytokine was lowered again. Throughout the time period under investigation, the major portion of CSF-1 mRNA encoded the secreted protein, while only a minor portion encoded the membrane-bound molecule.

MATERIALS AND METHDOS

Materials

Collagenase type II and alpha modified essential medium (α-MEM) were obtained from Seromed (Munich, Germany). Guanidine isothiocyanate was purchased from Gibco/BRL (Basel, Switzerland), and sarksoyl and heparin from FLUKA (Buchs, Switzerland). DNAse was purchased from Gene Hunter (Nashville, TN, U.S.A.) and the oligonucleotides were synthesized by Amplimmun (Madulain, Switzerland). The PCR Kits were purchased from Perkin-Elmer (Branchburg, NJ, U.S.A.). The TA cloning kit came from Invitrogen (Carlsbad, CA, U.S.A.) and the Klenow fragment from Boehringer Mannheim (Mannheim, Germany). T4 kinase and T4 DNA ligase were obtained from Promega (Madison, WI, U.S.A.). [γ-^{32}P]ATP was bought from Amersham (Buckinghamshire, U.K.) and the GeneScreen nylon membrane from NEN (Boston, MA, U.S.A.).

Cell culture

Primary osteoblastic cells were released from murine calvariae by sequential collagenase digestion.[25] Thirty dissected calvariae were incubated in a shaking waterbath for 30 minutes at 37°C in 3 ml of phosphate-buffered saline without Ca^{2+}/Mg^{2+}, containing 4 mM EDTA, pH 7.2. The cells were released by digestion with collagenase Type II, concentration 3 mg/ml in MEM/Hank's, for 5×20 minutes. Fractions 3–5 express osteoblastic characteristcs. Total RNA for RT-PCR was prepared from these cells.

The murine cell line MC3T3-E1,[26] kindly provided to us by Dr. Kodama, Ohu University School of Dentistry, Koriyama, Japan, was grown in α-MEM with Earle's salts, supplemented with 10% fetal bovine serum.

Preparation of RNA

Total RNA from cells and from metatarsal bones was isolated after lysis of cells and tissues with guanidine isothiocyanate.[27] Metatarsals II–IV from each paw from 12 fetuses or newborn mice were excised, pooled, transferred immediately into 200 μl of denaturing solution (8.5 M guanidine thiocyanate/25 mM Na-citrate pH 7.0/0.5% sarkosyl/100 mM β-mercaptoethanol), and homogenized in an Eppendorf tube with a Teflon homogenizer. Thereafter, another 200 μl of denaturing solution was added. In sequence, 40 μl of 2 M NaOAc, pH 4.5, 400 μl of water-saturated phenol, and 80 μl of chloroform/2% isoamylalcohol were added. After incubation for 15 minutes on ice, the samples were centrifuged, the aqueous phase was transferred into new tubes, and nucleic acids were precipitated with 50 μl of 8 M NH_4OAc and 1 ml of ethanol at –20°C. After centrifugation, RNA was redissolved in 500 μl of denaturing solution, reprecipitated, and washed in 70% ethanol. Before PCR, the RNA was treated with DNAse to remove contaminating DNA. For this purpose, ~20 μg of total RNA was dissolved in 50 μl of water. After addition of 5.7 μl of 10× reaction buffer and 10 U of DNAse, the mixture was incubated for 30 minutes at 37°C. The mixture was extracted with 40 μl of phenol:chloroform:isoamyl al-

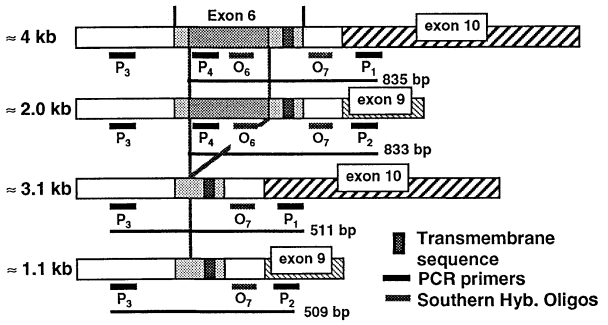

FIG. 1. Strategy for PCR. The four major transcripts encoding CSF-1 are schematically represented. Location of the PCR primers (P_1–P_4) and the respective amplicons are indicated. Oligonucleotides O_6 and O_7 were used for Southern hybridization, O_6 hybridizing to the amplicons derived from the transcripts encoding the secreted protein only, while O_7 hybridized to all amplified fragments. (Drawn after Stanley ER 1994 CSF-1 (macrophage colony-stimulating factor). In: Thomson A (ed.) The Cytokine Handbook, 2nd Ed. Academic Press Ltd., New York, NY, U.S.A., pp. 387–418.)

cohol, 25:24:1, and subsequently RNA was precipitated as described above.

Oligonucleotides

Locations of the oligonucleotides used for the amplification of fragments specific for CSF-1 mRNAs are schematically shown in Fig. 1.[28] The primers were chosen to allow to distinguish among the four previously described major transcripts encoding CSF-1. Primers P_1 and P_2 hybridize to sequences 1858–1877 in exon 10 and 1856–1875 in exon 9, respectively. The sequence of P_1 is 5′-CTTCCCATATGTCTCCTTC C-3′ and of P_2 5′-CAGAGGGACATTGACAAA CG-3′. Primers P_3 and P_4 hybridized to position 481–502 in exon 5 and 1042–1062 in exon 6, respectively. The sequence of P_3 is 5′-GCTTTGCTAAGTGCTCTAGCCG-3′ and of P_4 5′-GAATCTTCACTGGGCACTAAC-3′. To confirm the identity of the amplified fragments, Southern hybridization with internal oligonucleotides was performed. For this purpose, oligonucleotide O_6, hybridizing with nucleotides 1174–1194 in exon 6, and oligonucleotide O_7, hybridizing with nucleotides 1645–1665 in exon 7, with the respective sequences 5′-CCCAGGGCCCTCTCAGCATC-3′ and 5′-CACCTGCTGGTGCCGGGCATC-3′ were chosen.

The primers for murine osteopontin were 5′-CATGGACGACGATGATGACG-3′ at position 318–337 and 5′-TGCCCTTTCCGTTGTTGTCC-3′ at position 675–694.[29] To amplify a ~300 bp fragment from glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the primers 5′-CTTGTCATCAACGGGAAGC-3′ at position 239–257 and 5′-CAAAGTTGTCATGGATG ACC-3′ at position 526–545 were chosen. The internal primer for the GAPDH fragment had the sequence 5′-CCATCACAAACATGGGGGC-3′ and was located at position 425–406.[30]

Reverse transcription polymerase chain reaction

RT-PCR was performed according to the recommendations of the manufacturer of the RNA-PCR kit. Briefly, 50 ng of total RNA was denatured for 10 minutes at 75°C. Thereafter, a master mix containing $MgCl_2$, reaction buffer, nucleotides, random hexamers (50 μM), RNAse inhibitor (1 U/μl), and MuLV reverse transcriptase (2.5 U/μl) was added. Sample mixtures were allowed to stand at room temperature for 10 minutes before the start of the reverse transcription, which lasted for 30 minutes at 37°C. To start the PCR reaction, a master mix containing $MgCl_2$, reaction buffer, 0.5 U AmpliTaq DNA polymerase, and the respective primers at a final concentration of 0.1 μM were added to the cDNA. The PCR was performed for 30 cycles of 40 s at 94°C, 45 s at 50°C, and 1 minute at 72°C. At the end of the reaction, the samples were incubated for another 7 minutes at 72°C to allow completion of the synthesis of noncomplete DNA strands.

Construction of standards for quantitative PCR

The quantitative PCR system used in this study is based on the competitive amplification of exogenously added standard fragments and the sequences in the sample. To ensure the same efficiency of amplification during the PCR, the standards have to correspond both in size and sequence to the sequence which will be amplified from the sample. In addition, both standard and sample amplicons are amplified with the same primer pair. Thus, the four major CSF-1 fragments (see Fig. 1) were amplified and ligated into PCR (Invitrogen). The four plasmids were reverse amplified such that a portion of ~50 nucleotides was deleted in the center of the individual CSF-1 fragments and a new *Xba*I restriction site was introduced.[22,31] The following primers were used: for fragments derived from the transcripts encoding the secreted CSF-1, primers of the sequences 3′-CACGAAGGGTTTT CGGTG **AGATCT**ATGA-5′ and 3′-GAGGAAGCTAGGGACCAT**AGATCT**ATGA-5′, hybridizing at positions 1419–1437 and 1485–1503, respectively, were used (bold letters indicate the *Xba*I restriction site within the 5′-tail). For the fragments derived from the transcripts encoding the membrane-bound form of the cytokine, primers of the sequences 3′-CGGCTAGAGCACTTAGC**AGATCT**ATGA-5′ and 3′-CCTACTACGGGCCGTGGTC**AGATCT**ATGA-5′, hybridizing at positions 680–697 and 1651–1670 were used. After reverse amplification, the fragments were blunt-ended with Klenow fragment and phosphorylated at the 3′-ends with T4 kinase. The linear DNA was self-ligated with T4 DNA ligase.

Quantitative RT-PCR

To quantitate the individual transcripts encoding CSF-1, a set of PCRs was performed for each primer pair. Aliquots of 50 ng of sample RNA were reverse transcribed. After RT, different amounts of a standard plasmid, specific for each primer pair, were added and the cDNA was amplified together with the exogenously added standard. Thus, for each amplicon, a set of PCR assays was performed, each tube containing equal amounts of sample cDNA but differ-

ent numbers of standard molecules. Standard plasmid and cDNA compete during the PCR cycles and therefore low amplification of sample sequences is achieved in the presence of high copy numbers of standard molecules, while low copy numbers of the standard allow high amplification of sample sequences. The copy number of CSF-1 cDNAs equals the number of standard DNA molecules, when the amounts of the amplified fragments are equal.

Southern hybridization

To verify the identity of the amplified PCR fragments, and for their quantitation, Southern hybridization with [^{32}P]labeled oligonucleotides was performed.[32] Fifty nanograms of the respective nucleotide was labeled with T4 polynucleotide kinase in the presence of [γ–^{32}P]ATP. PCR fragments were separated on a 2% agarose gel, transferred onto a GeneScreen nylon membrane in 2× SSC, and the membrane was baked for 2 h at 80°C. The membranes were prehybridized for 2 h at 42°C in 5× SSC/0.1% SDS/5× Denhardt/0.1% NaPPi/0.5 mg of heparin/ml. Hybridization was performed at 42°C overnight in the same buffer, but 10^6 cpm of labeled probe were added. After hybridization, the nylons were washed at room temperature in 0.1× SSC/1% SDS for 3 × 30 minutes, the moist membranes were exposed to a PhosphorImager screen (STORM, Molecular Dynamics, Sunnyvale, CA, U.S.A.), and subsequently quantitated.

RESULTS

Quantitative RT-PCR for CSF-1

The transcripts encoding either the secreted or the membrane-bound molecular forms of CSF-1 were quantitated by qPCR. For this purpose, aliquots of total RNA were reverse transcribed. After the RT reaction, different amounts of standard plasmid, specific for each transcript, were added to the individual reactions. The amplified fragments were digested with the restriction endonuclease *Xba*I, separated by agarose gel electrophoresis, transferred onto a nylon membrane, and quantitated on a PhosphorImager after Southern hybridization with an internal oligonucleotide.[22,31] To standardize the PCR for the amount of RNA at the onset of the experiment, a fragment for GAPDH was coamplified with the CSF-1 fragments using a second primer pair (Fig. 2).

Expression of CSF-1 transcripts in primary murine calvaria cells

First, the relative amounts of the major CSF-1 transcripts in osteoblastic primary calvaria cells (pooled fractions 3–5) were determined by qRT-PCR. In agreement with Northern hybridizations (data not shown) it was found that the 4.0 kb transcript, encoding the secreted cytokine, was most abundant (Fig. 3). The transcript with the lowest copy number was the putative 1.1 kb mRNA, which is encoding the membrane-bound cytokine. Though it is possible that the different transcripts are translated with varying efficiencies, these numbers reflect the results that were previously found by immunoprecipitation.[21]

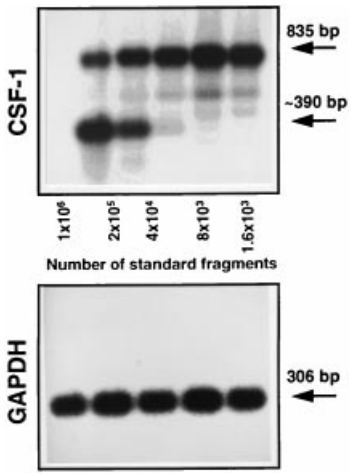

FIG. 2. Quantitative RT-PCR. Quantitative RT-PCR was performed as described in the Materials and Methods. Reverse transcription was performed in five reaction vessels, containing 50 ng of total RNA each. After RT, decreasing numbers of standard plasmid molecules, containing the standard fragment specific for the primer pair P_1/P_4, were added to the individual reaction mixes. The top panel shows the competition between the standard fragments (size after *Xba*I digestion ~390 bp) and the fragments derived from the 4.0 kb transcript (835 bp) during amplification. Subsequent Southern hybridization was performed with O_6. In parallel, RT-PCR for GAPDH was performed in each tube (bottom panel). This reaction was used as a control for the reproducibility of the RT-PCR reaction and the standardization of the amount of RNA at the beginning of the experiments.

Expression of CSF-1 transcripts in developing murine metatarsals

To determine whether there is a change in the expression of CSF-1 during the development of fetal murine metatarsals, total amounts of CSF-1 transcripts as well as the amounts of the individual transcripts were determined. Only a few transcripts encoding CSF-1 could be detected in 50 ng of total RNA from metatarsals from 15-day-old embryos (Fig. 4). Expression of CSF-1 transcripts was significantly increased in metatarsals from 16- and 17-day-old fetuses. A further significant increase of transcript levels were observed at 18 days. In RNA from metatarsals from newborn animals, the amount of transcripts encoding CSF-1 was again decreased as compared with the levels at 18 days.

In a next step, the distribution of the four major transcripts encoding CSF-1 was investigated in the metatarsals. The 4.0 kb transcript was the most prominent transcript in the RNA from metatarsals from 16-, 17-, and 18-day-old embryos as well as from newborn mice, ranging from 75% to 90% of total CSF-1 transcripts (Fig. 5). In the metatarsals from 15-day-old embryos, however, the 4.0 kb transcript,

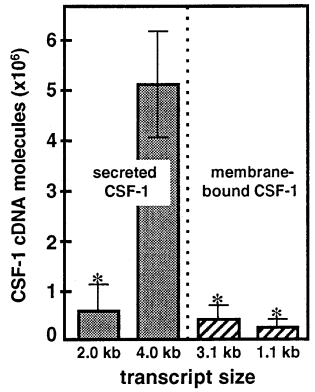

FIG. 3. Quantitative RT-PCR with total RNA from primary murine osteoblasts. Fifty nanograms of total RNA from primary murine osteoblastic cells was reverse transcribed and all four transcripts encoding CSF-1 were quantitated. As can be seen, the majority of CSF-1 is encoded by the 4.0 kb transcript, encoding the secreted cytokine. Only relatively few copies of each of the three other transcripts could be detected. The experiment was repeated three times and results are given as number of copies ± SEM (*$p < 0.05$).

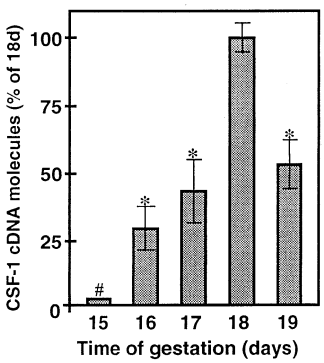

FIG. 4. Total CSF-1 transcripts in metatarsals. Transcripts encoding CSF-1 were determined in total RNA from murine metatarsals from 15-day-old embryos up to newborn mice. While only low levels of transcripts encoding the cytokine could be detected in 15-day metatarsals, the levels increased until 18 days. Thereafter, in metatarsals from newborn animals, levels of transcripts encoding CSF-1 dropped again. The experiment on the expression of transcripts encoding CSF-1 in fetal metatarsals was performed three times. Values are given as percentage from the number of transcripts in the metatarsals from the 18-day-old embryos ± SEM. #Significanly lower number of CSF-1 transcripts as compared with 16–19 days after gestation ($p < 0.05$). *Significantly lower number of CSF-1 transcripts as compared with 18 days after gestation ($p < 0.05$).

encoding the secreted form of the cytokine, makes up about 50% of total CSF-1 transcripts only. At this age, the 3.1 kb transcript, encoding the membrane-bound protein, is very prominent (between 27% and 56% in the individual experiments). The 3.1 kb transcript is present at considerably lower levels at the other ages tested (at day 16 between 0%

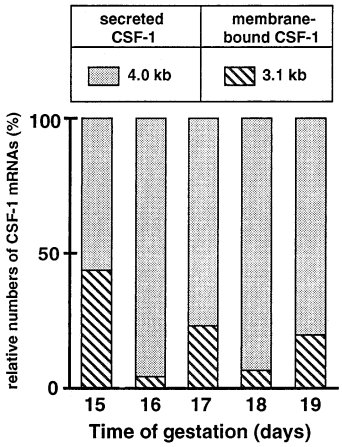

FIG. 5. Transcripts encoding CSF-1 in metatarsals. In murine metatarsals from 15-day-old embryos up to newborn mice, the four major transcripts encoding CSF-1 were determined. In metatarsals from 15-day-old embryos, where only low levels of transcripts could be detected, the 3.1 kb transcript, encoding the membrane-bound cytokine was most prominent. At all other ages, the 4.0 kb transcript, encoding the secreted molecule, was expressed at the highest level, followed by the 3.1 kb transcript, which was expressed between 5% and 20% only. The 2.0 kb and 1.1 kb transcripts were expressed with low copy numbers throughout the experimental period and are not shown in the figure. The experiment on the expression of transcripts encoding CSF-1 was performed three times.

and 8%; at day 17 between 15% and 32%; at day 18 between 0% and 10%; and in metatarsals from newborn mice between 7% and 30%). Only low levels of the transcripts of 2.0 kb (encoding the secreted protein) and of 1.1 kb (encoding the membrane-bound cytokine) could be detected during the period under study and are therefore not included in Fig. 5.

RT-PCR for osteopontin

As mentioned before, osteoblasts and chondrocytes are the major producers of CSF-1 in vivo. To determine whether the low levels of CSF-1 transcripts in metatarsals from newborn mice was due to an overall decrease of transcripts encoding osteoblastic and chondrocytic products, RT-PCR for osteopontin, an extracellular matrix protein expressed by these cells, was performed (Fig. 6). A 376 bp fragment derived from osteopontin transcripts was detected in metatarsals of all ages between 15-day-old embryos and newborn animals. The intensity of the signal after hybridization with an osteopontin cDNA increased with age, demonstrating that the levels of transcripts specific for osteoblasts and chondrocytes did not generally decrease. Constant levels of a ~300 bp fragment derived from GAPDH transcripts ensured that equal amounts of RNA were used in the reaction.

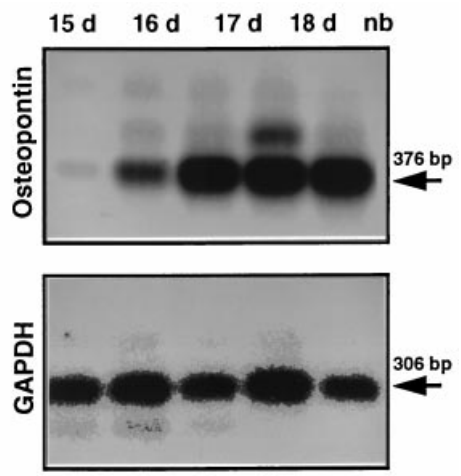

FIG. 6. Determination of osteopontin mRNA. To investigate whether the decline in CSF-1 mRNA expression in metatarsals from newborn mice was due to a general decrease in transcription of osteoblastic and chondrocytic products, mRNA levels for osteopontin were checked. For this purpose, RT-PCR was performed with 1 ng of total metatarsal RNA. The levels of mRNA encoding osteopontin increased throughout the experimental period (top panel), while GAPDH, which was used as a standard, remained constant (bottom panel).

DISCUSSION

Within this study, transcripts encoding the secreted or the membrane-bound forms of CSF-1 were quantitated in primary osteoblasts and in developing murine metatarsals. In primary osteoblasts from murine calvariae, the major portion of CSF-1 mRNA encodes the secreted form of the protein, while the transcripts encoding the membrane-bound cytokine represent a minor fraction of total CSF-1 mRNA. This finding is in agreement with previous results describing that murine osteoblastic cell lines and primary calvaria cells produce both the secreted and the membrane-bound protein, the latter however, accounting for a minor portion of the cytokine only.[21] Thus, it can be assumed that protein levels are reflected by the abundance of the respective transcripts.

The investigation was prompted by the finding that the reversal of the osteopetrotic phenotype in *op*/*op* mice upon injections of CSF-1 was not complete.[8,9] A possible explanation for this observation is that circulating CSF-1 may not be able to mediate the whole array of effects, and locally produced CSF-1 might be essential for full biological action. Indeed, in bone, CSF-1 transcripts were detected in cells located in close proximity to the sites of osteoclast formation and bone resorption,[12] suggesting a role for locally produced and acting cytokine in these processes. This hypothesis was further supported by data describing that glutaraldehyde-fixed layers of normal osteoblasts, or stromal cells were able to support the growth of the CSF-1–dependent macrophage cell line BAC-1[21] and the formation of osteoclasts from hemopoietic precursors.[33,34] Thus, the cytokine incorporated into the cell layer, either exposed on the cell surface or bound to the extracellular matrix, is sufficient for proliferation and/or differentiation of CSF-1 responsive cells. Furthermore, cocultures of spleen cells together with osteoblasts from *op*/*op* animals required high concentrations of exogenous CSF-1 for the formation of osteoclasts,[13] suggesting the soluble form to be less efficient in supporting this process.

The demonstration that in vivo during bone development both the membrane-bound and the secreted proteins are synthesized indicates that both molecular forms of the cytokine play a role in this organ. The effects exerted by the cytokine may depend on its local concentrations, which are difficult to assess. But it is tempting to speculate, based on the relatively high amount of transcripts encoding the membrane-bound protein in younger embryos that this form plays a decisive role in the attraction, proliferation, and/or differentiation of early osteoclast progenitors.

Highest levels of transcripts encoding CSF-1 were determined in metatarsals from 18-day-old fetuses. Several processes that may be influenced by CSF-1 take place in these bones, namely fusion of osteoclast precursors, and migration and activation of mature osteoclasts. The cytokine has previously been shown to be required for the formation of multinuclear osteoclasts from precursor cells.[35] Other reports, however, suggested that the presence of CSF-1 might not be required for fusion of osteoclast precursors to occur, but is essential for the proliferation of the cells of the osteoclast precursor pool.[36] These latter results were recently supported by the finding that macrophages and osteoclasts develop also in the absence of CSF-1, when apoptosis of the precursors is prevented.[37] The role of the cytokine might therefore be limited to ensure survival of the precursor cells of the macrophage and osteoclast lineages, the cells developing independently of exogenous growth factor along a predetermined differentiation pathway. This limited action of CSF-1 during the formation of osteoclasts may also explain, at least in part, the observed spontaneous recovery of osteopetrotic *op*/*op* mice 6–8 weeks after birth.[38,39] In these animals, precursors may be present either due to maternal exchange of CSF-1 through the placenta during pregnancy or due to a failure of apoptosis in osteoclast precursor cells. A last possibility might be the formation of osteoclasts due to residual CSF-1 activity in *op*/*op* animals[40] or due to the action of granulocyte macrophage-CSF (GM-CSF) on osteoclast precursors.[41–43] In cell culture experiments, CSF-1 was demonstrated to exert effects also on the mature osteoclasts. The cytokine inhibited the activity while stimulating migration of these cells.[14,16] Thus, although the actual role of the cytokine is not yet fully understood, the high levels of CSF-1 transcripts in the metatarsals from 18-day-old embryos may ascertain survival and differentiation of osteoclast precursors and may be involved in the regulation of the resorption activity of the mature cells and of the distribution of the resorption sites.

The number of CSF-1 transcripts slightly decreased again in metatarsals from newborn animals. This decline in CSF-1 mRNA however, is not due to a general decrease in mRNA, since levels of transcripts encoding osteopontin, an extra-

cellular matrix protein expressed by cells of the osteoblastic lineage and by hypertrophic chondrocytes, are increasing throughout the investigated time frame.[44] While this decrease in transcript levels may not be relevant for the respective protein levels, it can be envisaged that the bone-resorbing osteoclasts require lower amounts of CSF-1 for survival than precursor cells need for proliferation and differentiation. In addition, cytokine released from the bone matrix during resorption may substitute for newly synthesized protein, a theory that has been put forward to explain the potential biological roles of cytokines incorporated into the extracellular matrix.

Until now, it is not clear whether there is a differential regulation of the transcripts containing either exon 9 or exon 10 of the CSF-1 gene in their 3′-UTR. It was suggested that exon 10 contains purine reach regions[28] that have been shown in other transcripts, such as GM-CSF mRNA, to confer RNA instability.[45] Our data show that during the period under investigation, only a minor fraction of CSF-1 transcripts makes use of exon 9. Thus, if mRNA stabilization is critical for the regulation of CSF-1 expression during the observed time period, other mechanisms than differential usage of the 3′-UTR exons 9 and 10, respectively, are employed.

In conclusion, CSF-1 is mainly synthesized as a secreted molecule which acts either directly in a paracrine manner or may be integrated into the extracellular matrix. Though it is evident from the reconstitution experiments, in which CSF-1 was injected into osteopetrotic *op*/*op* animals, that locally produced CSF-1 is critical for normal bone resorption, the contribution of the secreted and the membrane bound forms in this process is not clear. The present results, however, demonstrate that in vivo, the membrane-bound cytokine is synthesized during the formation of osteoclasts and thus the lack of this molecular form may be responsible for the incomplete reversal of the osteopetrotic phenotype in CSF-1–treated *op*/*op* mice.

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