COSMO BIO CO., LTD.
Inspiration for Life Science

POLYCLONAL ANTIBODY

For research use only. Not for clinical diagnosis.

Catalog No. LSL-LB-4225

Anti Osteopontin

Product type	Primary antibodies
Host	Rabbit
Source	Serum
Form	Liquid This product does not contain preservatives such as NaN3.
Volume	100µl
Concentration	
Specificity	Does not cross react with Type I, II, III, IV, V, VI collagen, laminin and fibronectin. MAP sequence match 17 out of 17 amino acids of the rat, 14/17 aa of the bovine, 15/17 aa of the human.
Antigen	Mouse multiple antigen peptide
Isotype	

Application notes

ELISA, WB, IHC, IF

Recommended use

According to the ELISA assay, results are positive for dilutions up to 200,000 fold against linear synthetic peptides.

Immunohistochemistry on Frozen or Paraffin section

Recommended dilutions

Immunofluoresence; More than 1/50 (recommended: 1/50～1/500)

Western Blotting; More than 1/500

Optimal dilutions/concentrations should be determined by the end user.

Staining Pattern

Appears as a single band by Western blotting.

Represent high molecular weight aggregates (Robey, 1996). The anti-osteopontin antibody shows a band at 56 kDa and a smearing at the top of the lane (lane 3).

The smearing probably represents high molecular weight aggregates (Sørensen et al., 1994; Rittling et al., 1998). Major bands of BSP are present.

Cross reactivity

Cross react with mouse, rat, bovine and human.

The MAP contains the SSE sequence of mouse Osteopontin.

Cross reaction will differ between species.

Storage

Store below -20°C (below -70°C for prolonged storage). After thawing, store in small aliquots in sealable vials and store below -70°C. To prevent degradation from repeated thawing, store the antiserum between 0 to 4°C after second thawing.

Stable for three years at -70°C

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3) Rittling, S.R. et al., Mice lacking osteopontin show normal development and bone structure but display altered osteoclast formation in vitro. J. Bone Miner. Res. 13:1101-1111.

4) Sørensen, E.S. et al., Localization of transglutaminase-react glutamine residues in bovine osteopontin. Biochem. J. 304:13-16, 1994.

5) Termine, J.D. and Robey, P.G.: Bone matrix proteins and the mineralization process. In: Primer on the metabolic bone diseases and disorders of mineral metabolism, ed. by Favus, M.J., Lippincott-Raven, Philadelphia, 1996, PP.24-28.

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LSL

#LB-4225 Anti (MAP) Osteopontin

を用いてWB

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Pinero, G.J., Farach-Carson, M.C., Devoll, R.E., Aubin, J.E., Brunn, J.C. and Butler, W.T.: Bone matrix proteins in osteogenesis and remodelling in the neonatal rat mandible as studied by immunolocalization of osteopontin, bone sialoprotein α_2HS-glycoprotein and alkaline phosphatase. Archs. oral. Biol. 40:145-155, 1995.

✓ Rittling, S.R., Matsumoto, H.N., McKee, M.D., Nanci, A., An, X.-R., Novick, K.E., Kowalski, A.J., Noda, M. and Denhardt, D.T.: Mice lacking osteopontin show normal development and bone structure but display altered osteoclast formation in vitro. J. Bone Miner. Res. 13:1101-1111.

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Anat Embryol (2000) 202:31–37

ORIGINAL ARTICLE

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Expression of major bone extracellular matrix proteins during embryonic osteogenesis in rat mandibles

Accepted: 21 December 1999

Abstract It is not known how bone proteins appear in the matrix before and after calcification during embryonic osteogenesis. The present study was designed to investigate expressions of the five major bone extracellular matrix proteins – i.e. type I collagen, osteonectin, osteopontin, bone sialoprotein and osteocalcin – during osteogenesis in rat embryonic mandibles immunohistochemically, and their involvement in calcification demonstrated by von Kossa staining. Wistar rat embryos 14 to 18 days post coitum were used. Osteogenesis was not seen in 14-day rat embryonic mandibles. Type I collagen was localized in the uncalcifed bone matrix in 15-day mandibles, where no other bone proteins showed immunoreactivity. Osteonectin, osteopontin, bone sialoprotein and osteocalcin appeared almost simultaneously in the calcified bone matrix of 16-day mandibles and accumulated continuously in 18-day mandibles. The present study suggested that type I collagen constitutes the basic framework of the bone matrix upon which the noncollagenous proteins are oriented to lead to calcification, whereas the noncollagenous proteins are deposited simultaneously by osteoblasts and are involved in calcification cooperatively.

Key words Bone · Calcification · Type I collagen · Noncollagenous proteins · Immunohistochemistry

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Introduction

The bone matrix is composed of organic and mineral components. Type I collagen is a primary gene product of osteoblasts during bone matrix formation (Aubin and Liu 1996) and comprises 85% to 90% of the total organic bone matrix (Termine and Robey 1996). The most abundant noncollagenous protein produced by osteoblasts is osteonectin, a phosphorylated glycoprotein that has high affinity for binding ionic calcium and physiologic hydroxyapatite (Termine and Robey 1996). Osteopontin is a phosphorylated glycoprotein secreted by osteoblasts and has been suggested to occur at an early stage during bone development and to facilitate attachment of osteoblasts to the extracellular matrix (Butler 1989; Butler et al. 1996). Bone sialoprotein (BSP) is an osteoblast-derived heavily glycosylated protein of the bone matrix expressed at late stages of differentiation (Bianco et al. 1991) and its appearance is tightly correlated with the appearance of mineral (Bianco et al. 1993). Osteocalcin is a γ-carboxy glutamic acid-containing protein that has been considered the latest of expression markers in mature osteoblasts (Aubin and Liu 1996).

Distribution of the bone matrix proteins in embryonic and infant bones has been reported using immunohistochemistry for osteopontin and ostecalcin in rats (Mark et al. 1988), osteocalcin in human (Ohta et al. 1989), osteopontin and osteonectin in pigs (Chen et al. 1991) and osteopontin and BSP in rats (Pinero et al. 1995). The gene expression of the bone matrix components in embryos and infants has been reported for BSP in rats (Chen et al. 1992), osteonectin,osteopontin and osteocalcin in mice (Nakase et al. 1994), and osteonectin, osteopontin, BSP and osteocalcin in mice (Sommer et al. 1996). However, it is not known how the gene products of the major five bone proteins, i.e. type I collagen, osteonectin, osteopontin, BSP and osteocalcin are sequentially deposited and accumulated extracellularly during embryonic osteogenesis in a single animal model and how the maturation of the bone organic matrix is involved in calcification.

Most of the rat mandibular bone is formed through intramembranous ossification (Savostin-Asling and Asling 1973) and can be used as a convenient model to follow osteogenesis and maturation of the bone matrix without any interference with the cartilage matrix. We hypothesized that the major bone proteins are deposited and accumulate sequentially in the extracellular environment during embryonic osteogenesis to construct the mature bone matrix with calcification. To test the hypothesis, this study was designed to analyze the presence of the five major bone proteins during osteogenesis in the rat embryonic mandible immunohistochemically, and the concurrent calcification was demonstrated by von Kossa staining.

Materials and methods

Preparation of embryonic tissue

Wistar rat embryos 14- to 18-days post coitum (dpc) were used. Heads were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C overnight. After dehydration through a graded series of ethanol solutions, the tissues were embedded in paraffin. Serial sections were cut at 5 μm and adjacent sections were processed for von Kossa staining to identify calcium salt formation (Thompson and Hunt 1966), counterstained with haematoxylin-eosin, or used for immunohistochemistry for type I collagen, osteonectin, osteopontin, bone sialoprotein (BSP) and osteocalcin.

Preparation of antibodies

The antibodies were raised in rabbits against the synthesized peptide antigens selected from the rat osteonectin (-QNHHCKHGKV-CELDESNT-), rat osteopontin (-LKFRISHELESSSSE-), rat BSP (-KNFHRRIKAEDSEENGV-) and rat osteocalcin (-APYPDPL-EPHREVCELNP-) deduced from the respective cDNAs using the multiple antigen peptide (MAP) system (Tam 1988; Chung et al. 1995). A stepwise solid-phase procedure on *t*-butoxycarbonyl (Boc) βAla-OCH_2-Pam resin was accomplished manually in the presence of 0.05 mM of βAla in 0.5 g of resin. The synthesis of the first and every subsequent level of the carrier core was achieved using a 4 M excess of preformed symmetrical anhydride of diBoc-LysDCHA for a total of three cycles. The peptide of each antigen was extended to the MAP octa-branched matrix core using a peptide synthesizer. The synthetic peptide MAP was purified on HPLC columns and used as an antigen.

The antiserum against rat collagen type I was purchased from COSMO BIO/LSL (Tokyo, Japan).

Characterization of antibodies by western blotting

Noncollagenous proteins were extracted from the rat bone matrix by 0.5 M EDTA or 0.5 M EDTA and 4 M guanidine hydrochloride and subjected to electrophoresis in 5–20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (ATTO, Tokyo, Japan). The proteins from the bone matrix were blotted onto the PVDF membrane (ATTO) and stained with the antibodies against osteonectin, osteopontin, BSP and osteocalcin. The membrane was stained by indirect enzymatic immunochemistry of alkaline phosphatase-conjugated sheep anti-rabbit IgG (Boehringer Mannheim, Mannheim, Germany) and visualized by X-phosphate/NBT (Boehringer Mannheim). The results of the western blotting are shown in Fig. 1. The characterization of the anti-type I collagen antibodies using western blotting was described previously (Mizoguchi et al. 1990).

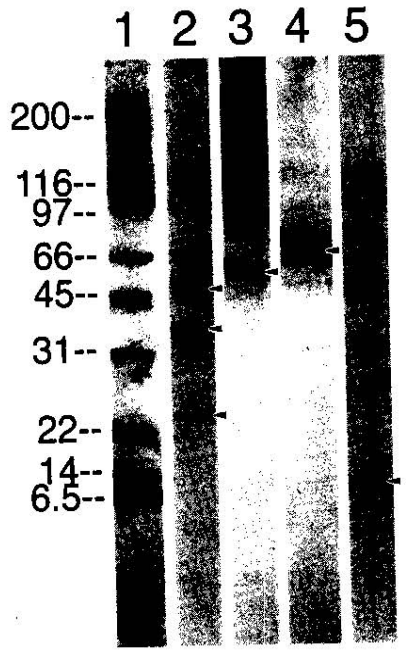

Fig. 1 Results of western blotting. The molecular weight markers are indicated in lane 1. The anti-osteonectin antibody reveals three major bands (▲) at 23 kDa, 36 kDa and 49 kDa (lane 2). The 36 kDa band is of osteonectin molecules and the 23 kDa may be of low molecular weight degraded products while the 49 kDa may represent high molecular weight aggregates (Robey 1996). The anti-osteopontin antibody shows a band (▲) at 56 kDa and a smearing at the top of the lane (lane 3). The smearing probably represents high molecular weight aggregates (Sørensen et al. 1994; Rittling et al. 1998). A major band (▲) of BSP is present at 65 kDa (lane 4; Robey 1996). A band of osteocalcin (▲) is at 10 kDa (lane 5; Gundberg et al. 1984)

Immunohistochemistry.

First, serial sections were cut, deparaffinized and treated with EDTA and/or enzymes as follows: For type I collagen and osteonectin immunohistochemistry, the sections were treated with 2.5% hyaluronidase from bovine testes (Sigma Chemical, St. Louis, Mo., USA) in phosphate-buffered saline (PBS) for 1 h at 37°C. For osteopontin, the sections were decalcified in 10% EDTA in 0.1 M phosphate buffer for 1 h at room temperature (RT), washed in PBS containing 0.025% Triton X-100 (PBS-TX) and treated with 2.5% hyaluronidase in PBS for 1 h at 37°C. For BSP and osteocalcin, the sections were decalcified in 10% EDTA in 0.1 M phosphate-buffered saline for 1 h at RT, washed in PBS-TX and treated with 0.01% protease type XXIV (Sigma) in PBS for 10 min at RT.

Second, after washing in PBS-TX, the sections were incubated with the antibodies against rat type I collagen, rat osteonectin, rat osteopontin, rat BSP or rat osteocalcin for 2 h at RT. The primary antibodies were diluted 1:200 with 5% normal goat serum in PBS-TX (NGS-PBS-TX) for the anti-type I collagen antibody, 1:100 for the anti-osteonectin, anti-osteopontin and anti-BSP, and 1:50 for the anti-osteocalcin antibodies.

Third, after washing in PBS-TX, the sections were incubated with the secondary antibodies, FITC-conjugated goat anti-rabbit F(ab) fragments of immunoglobulins (Biosource International, Camarillo, Calif., USA) for 1 h at RT. The antibodies were diluted 1:50 with 1% bovine serum albumin in PBS-TX.

Fourth, the sections were washed in PBS-TX, mounted in an aqueous mounting medium (Immunon Lipshaw, Pittsburgh, Pa., USA), and observed with a fluorescent microscope (Olympus AX80).

Fifth, control sections were processed routinely except that preimmune rabbit serum or NGS-PBS-TX was used as a substitute for the primary antibodies.

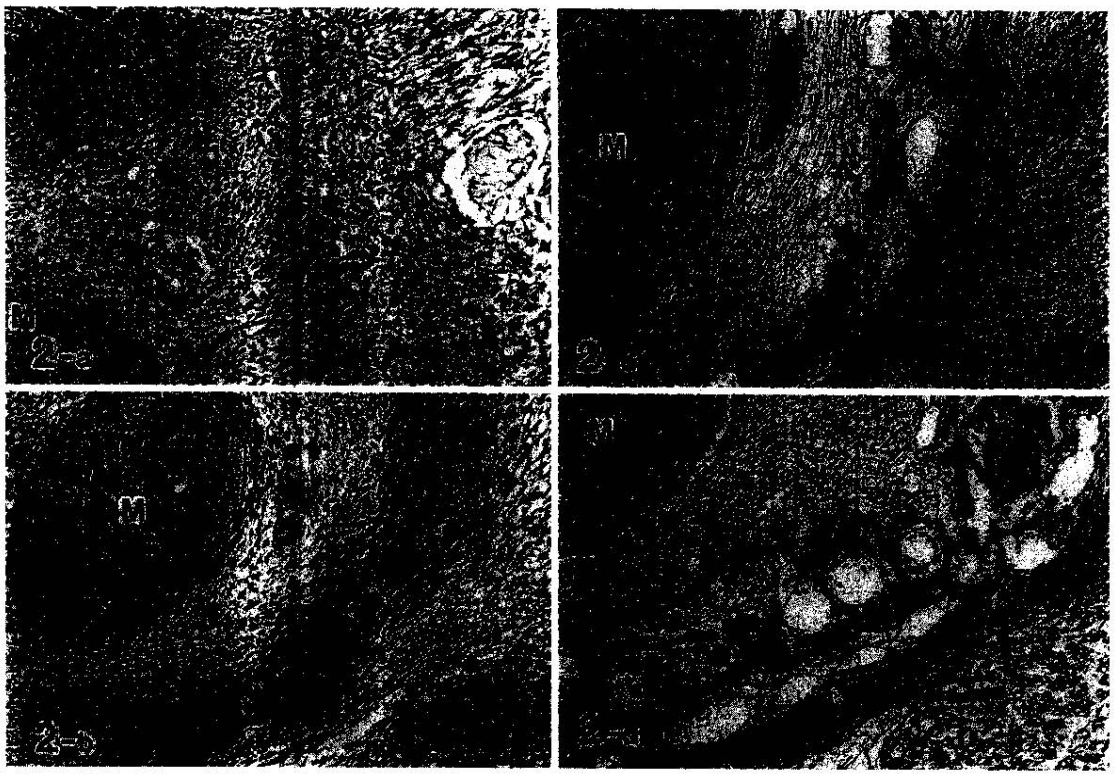

Fig. 2a–d von Kossa staining counterstained with haematoxylin-eosin. Mesenchymal cell condensation (*), assumed to be the putative osteogenic region, is formed in 14-day rat embryonic mandibles (E14; **a**). Uncalcified bone matrix (*) is deposited at E15 (**b**), and the bone matrix (*) becomes calcified at E16 (**c**). The calcified bone matrix (*) expands its area at E18 (**d**) *M* Meckel's cartilage. ×110

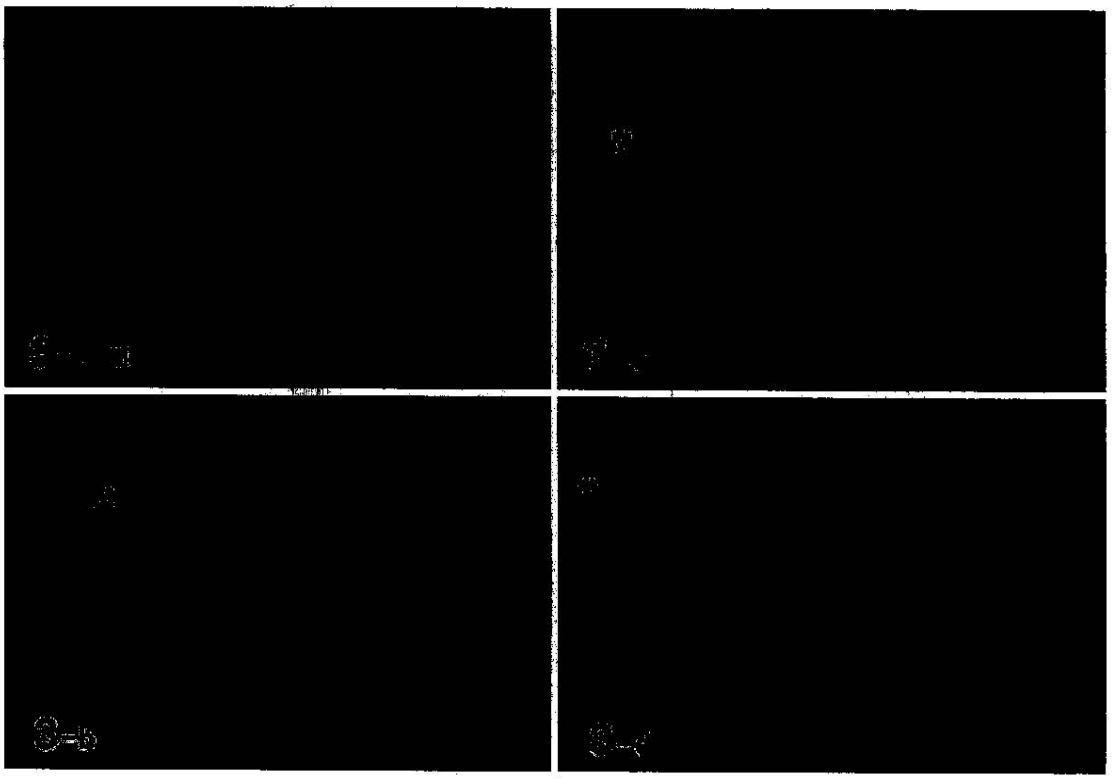

Fig. 3a–d Immunohistochemistry for type I collagen. Weak immunoreactivity is observed in mesenchymal tissue and Meckel's cartilage (*M*) at E14 (**a**). Immunoreactivity is localized in the uncalcifed bone matrix (*) at E15 (**b**). The calcified bone matrix (*), especially the peripheral region of the bone matrix at E16 (**c**) and E18 (**d**), is positive with the antibodies. The immunoreactivity in Meckel's carilage is no longer seen at E15 or older embryonic mandibles. ×110

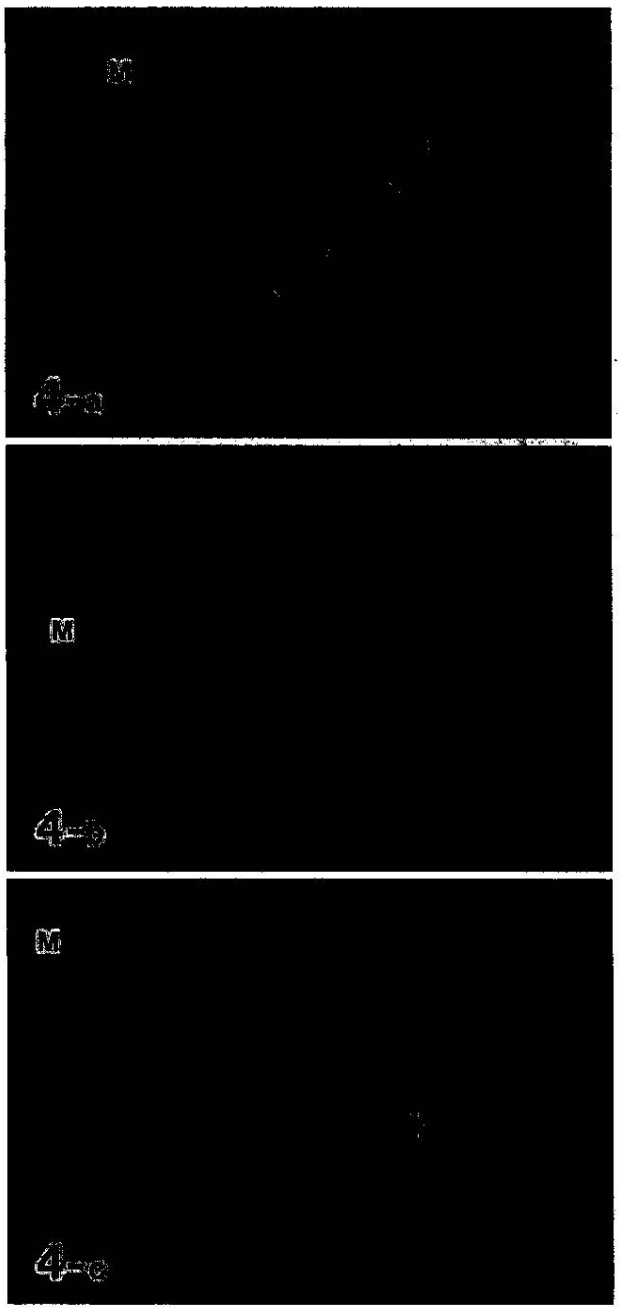

Fig. 4a–c Immunohistochemistry for osteonectin. Osteoblasts around the uncalcified bone matrix (▲) and chondrocytes of Meckel's cartilage (*M*) show immnunoreactivity at E15, whereas the uncalcified bone matrix is negative (**a**). The calcified bone matrix (*) is immunoreactive for osteonectin as well as osteoblasts and chondrocytes at E16 (**b**) and E18 (**c**). ×125

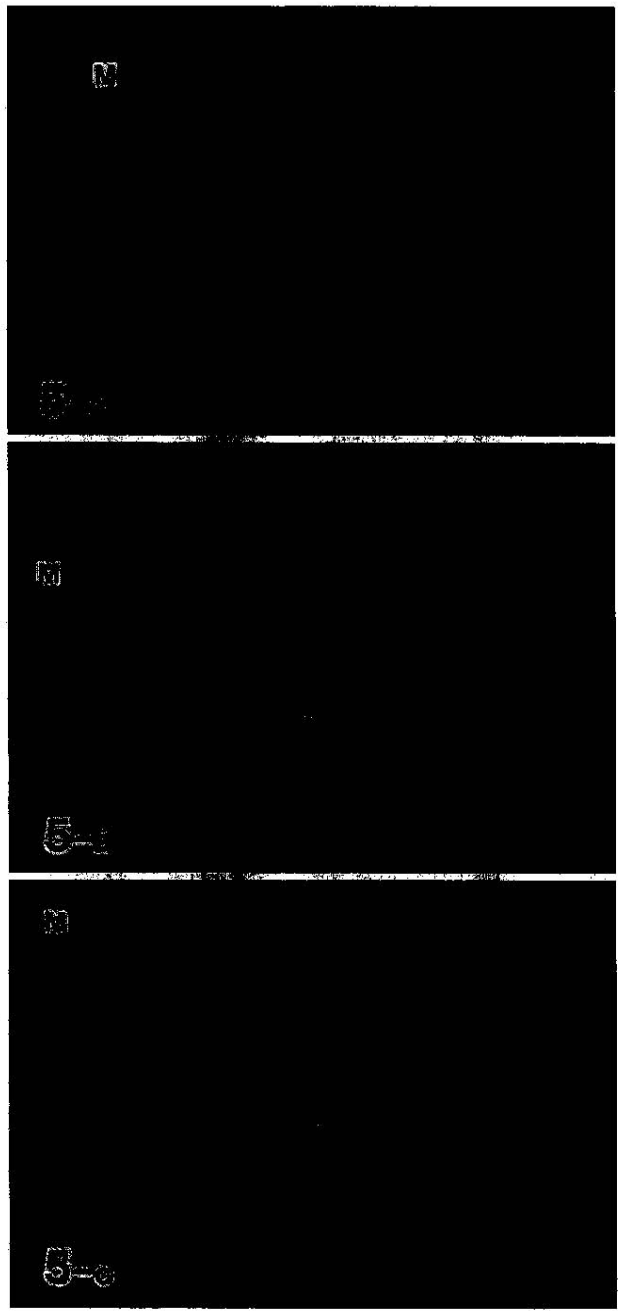

Fig. 5a–c Immunohistochemistry for osteopontin. No immunoreactivity is observed in the bone matrix (*) at E15 (**a**). The immunoreactivity is localized to the calcified bone matrix (*), especially the peripheral region of the bone matrix in E16 (**b**) and E18 (**c**). ×125

Results

A mesenchymal cell condensation, assumed to be the putative osteogenic region, was formed in 14-day (E14) rat embryonic mandibles (Fig. 2a). Bone matrix was deposited at E15 (Fig. 2b) and it became calcified at E16 (Fig. 2c), expanding its area at E18 (Fig. 2d).

Weak immunoreactivity for type I collagen was observed in mesenchymal tissue and Meckel's cartilage at E14 (Fig. 3a). Type I collagen was localized in the uncalcifed bone matrix at E15 (Fig. 3b). The calcified bone matrix, especially the peripheral region of the bone matrix at E16 (Fig. 3c) and E18 (Fig. 3d), was positive with the anti-type I collagen antibodies.

No immunoreactivity for osteonectin was seen at E14 (data not shown). Osteoblasts around the uncalcified bone matrix and chondrocytes of Meckel's cartilage showed immnunoreactivity for osteonectin at E15, whereas the uncalcified bone matrix was negative (Fig. 4a). The calcified bone matrix was immunoreactive for

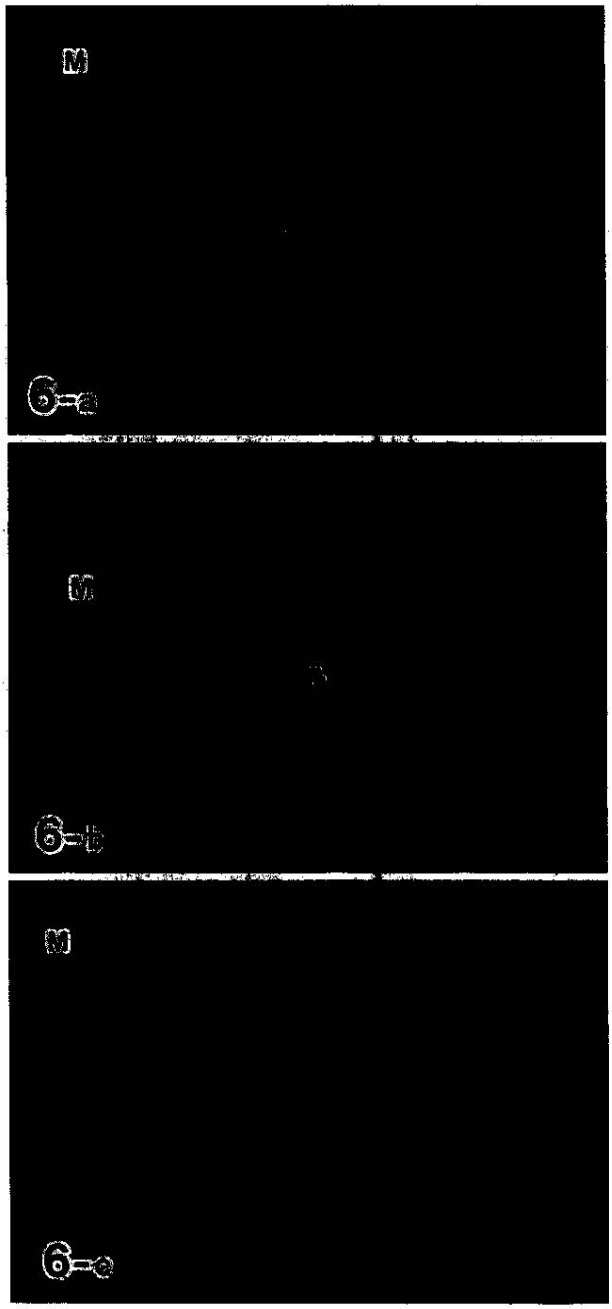

Fig. 6 Immunohistochemistry for BSP. No immunoreactivity is recognized in the bone matrix (*) at E15 (**a**). The immunoreactivity is specific to the calcified bone matrix (*) at E16 (**b**) and E18 (**c**). ×125

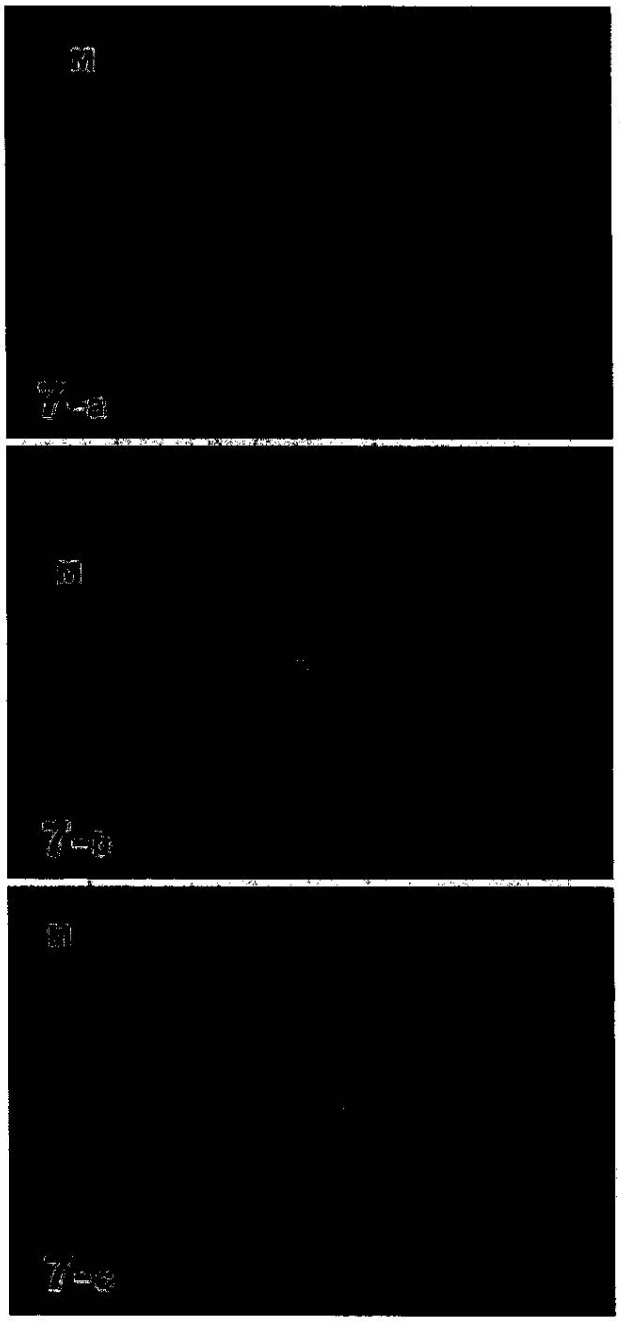

Fig. 7a–c Immunohistochemistry for osteocalcin. No immunoreactivity is recognized in the bone matrix (*) at E15 (**a**). The immunoreactivity is specific to the calcified bone matrix (*) at E16 (**b**) and E18 (**c**). ×125

osteonectin as well as osteoblasts and chondrocytes at E16 (Fig. 4b) and E18 (Fig. 4c).

No immunoreactivity for osteopontin was observed at E14 or E15 (Fig. 5a). The immunoreactivity was localized to the calcified bone matrix, especially the peripheral region of the bone matrix at E16 (Fig. 5b) and E18 (Fig. 5c).

No immunoreactivity for either BSP or osteocalcin was recognized at E14 or E15 (Figs. 6a,7a). Immunoreactivities with both antibodies were specific to the calcified bone matrix, as seen at E16 (Figs. 6b,7b) and E18 (Figs. 6c,7c).

None of the controls for immunohistochemistry showed labeling.

Discussion

Distribution of bone proteins in embryonic and infant bones has been investigated using immunohistochemistry (Mark et al. 1988; Ohta et al. 1989; Chen et al. 1991; Pinero et al. 1995) and in situ hybridization (Chen et al. 1992; Nakase et al. 1994; Sommer et al. 1996). Howev-

er, it is not known how the bone proteins appear in the matrix during embryonic osteogenesis in a single animal model before and after calcification. The present study demonstrated expression of the major bone proteins, i.e. type I collagen, osteonectin, osteopontin, BSP and osteocalcin during rat embryonic mandibular osteogenesis.

Type I collagen appeared in the osteogenic region in 15-day rat embryonic mandibles (E15) before calcification. Type I collagen constitutes the basic framework of the bone matrix upon which noncollagenous proteins are oriented to lead to calcification (Termine and Robey 1996). The immunoreactivity of type I collagen was also localized in Meckel's cartilage at E14, but no longer seen at E15 or older embryonic mandibles. The type I collagen protein could be transitionally expressed in immature Meckel's cartilage as previously reported (Sasano et al. 1992).

Osteopontin has been supposed to be an early marker of the bone matrix, whereas BSP and osteocalcin have been late markers (Aubin and Liu 1996; Butler et al. 1996; Termine and Robey 1996), and therefore osteopontin was expected to be deposited and accumulate extracellularlly before BSP and osteocalcin. However, such sequential expression of the noncollagenous proteins was postulated based on reviews of different studies using different experimental systems (Mark et al. 1988; Butler 1989; Bianco et al.,1991 1993; Helder et al. 1993). The present study first examined developmental expression of the major bone extracellular matrix proteins during embryonic osteogenesis using the same experimental model and indicated that, contrary to the previous expectation, osteopontin appear almost simultaneously with BSP and osteocalcin as well as osteonectin in the bone matrix at E16 concomitant with calcification. These proteins may be deposited in an orchestrated manner by osteoblasts to be involved in calcification cooperatively.

The present study showed that chondrocytes of Meckel's cartilage as well as osteoblasts and the bone matrix are immnunoreactive for osteonectin in rat embryonic mandibles. The gene (Nakase et al. 1994) and protein (Chen et al. 1991) expressions of osteonectin have been described in chondrocytes of cartilages that do not undergo calcification. Osteonectin in the chondrocytes may be involved in functions such as cell cycle regulation and modulation of shape change, rather than calcification (Termine and Robey 1996).

The present study suggested that the bone matrix proteins continue to accumulate in the matrix as embryonic osteogenesis proceeds and bone expands its area. At E16 and E18, type I collagen and osteopontin showed intense immunoreactivity in the peripheral region of bone, which consists of immature matrix components, whereas the immunoreactivity became less intense in the central, more mature region. It is possible that the epitopes of these proteins may be masked and/or degraded as the bone matrix mineralizes and matures.

The western blotting showed that the antibodies used in the present study are specific to each of the bone matrix proteins analyzed. We formulated our protocol for immunohistochemistry after testing effects of decalcification and enzymatic pretreatment. Decalcification of the sections in 10% EDTA was necessary to reveal immunoreactivity for osteopontin, BSP and osteocalcin. Mineral in the tissue may prevent the interaction of the antibodies with these proteins (Poole et al. 1982) In contrast, decalcification reduced immunoreactivity for osteonectin, some of which may have been lost from the section with calcium since osteonectin has high affinity for binding ionic calcium and physiologic hydroxyapatite (Termine and Robey 1996). Decalcification had no effect on immunoreactivity of type I collagen. The enzymatic pretreatments using hyaluronidase and protease were well established for immunohistochemistry of extracellular matrix proteins in our previous studies (Sasano et al. 1993, 1997, 1998). The hyaluronidase treatment was better to reveal the immunoreactivity for type I collagen, osteonectin and osteopontin whereas the protease was better for BSP and osteocalcin.

Further immunohistochemical investigation on developmental expression of extracellular matrix proteins during osteogenesis would provide a better understanding of maturation of the bone matrix.

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カタログNo. LB－4225

Anti (mouse MAP) Osteopontin

Ⅰ. 内容

Lot No. 716061

免疫動物	ウサギ
性状・包装ｻｲｽﾞ	全血清・１００μℓ
力価	ELISA で200,000 倍希釈まで陽性（合成直鎖ペプチドに対して）
抗原由来	マウス Multiple Antigen Peptide ＊
種間交差	マウス、ラット、ウシ、ヒトと交差＊
特徴	typeⅠ・Ⅱ・Ⅲ・Ⅳ・Ⅴ・Ⅵ collagen,laminin 及びfiblonectin と非交差。ラット、ウシ・骨粉のEDTA脱灰抽出物を用いた Western Blotting でosteopontinの分子量に相当する位置に陽性バンドを１本確認。
標準希釈率	蛍光抗体法で１：５０以上 (1/50～1/500) Western Blottingに関しては１：５００以上

（ ）内は推薦希釈率

＊種によって交差性が異なると思われます、希釈率等充分に検討下さい。
但し、ＭＡＰに合成したﾏｳｽ osteopontin 中のーＳＳＥーを含むアミノ酸配列（１７残基）はラットの同一部位のアミノ酸配列と１７／１７、ヒトと１５／１７、ウシと１４／１７一致している。

Ⅱ. 保存上の注意

－２０℃以下（長期間の場合は－７０℃以下）で凍結して下さい。
解凍後は密栓のできる小型容器に研究の規模に応じて少量づつ分注し、－７０℃以下で保存して下さい。
凍結融解の繰り返しによる力価の低下を避ける為、再解凍後の抗血清は０～４℃に保ち操作・保存して下さい。

Ⅲ. 安定性

－７０℃で３年間安定。
但し、NaN_3 等の防腐剤は入っていません。

製造元

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総発売元

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