INTRODUCTION

In bone pathologies and in bone regeneration it is important to assess changes in the composition of key proteins like osteocalcin, osteonectin, osteopontin and collagen (1, 2). For such functional description where conventional histology is insufficient, immunohistology may reveal additional information. However, immunohistology in general depends on factors like fixation and embedding material and technique (3, 4) and for bone especially the question of decalcification is an additional challenge (5). Routine fixation in pathology uses 4% formalin granting good morphological preservation on the cellular level (6). However, formalin fixation produces intermolecular bindings between proteins thus masking epitopes which may lead to a false negative immunostaining. Other fixatives are alcohol based, like methanol, ethanol or mixtures. They lead to a detailed tissue preservation, but are often linked with a strong but diffuse background staining which may irritate (7).

Paraffin, the standard embedding medium, is inert, a fundamental advantage to not interact chemically with the tissue (6). However, it can be applied to decalcified tissue only. Thus trabecular bone structures are only poorly preserved and essential information about localization and amount of mineralization e.g. in newly formed bone was eliminated. Shrinking artefacts on sections may lead to misinterpretations in particular in bone marrow. In contrast, for the assessment of lamellar substructure and cement lines (or reversal lines) (8, 9), decalcified bone samples should be preferred avoiding crack lines (6). Immunolocalization of the bone matrix protein markers like osteonectin (10) and osteocalcin (11) are already performed on sections of paraffin-embedded decalcified bone. Methyl-methacrylate has been used to embed non-decalcified bone for more than forty years delivering good histology (12). However, the preservation of enzyme activity and protein antigenicity of the tissue depend on the polymerization temperature (13, 14). A low-temperature pure MMA embedding at 4°C has been first described by Westen et al. (15). Since then different temperatures, catalysts, flexibilizers and accelerators have been tested (16, 17) to adapt MMA embedding resin to the characteristics of bone tissue. Optimal conditions for microsectioning and grinding are achieved, allowing for the various osseous cell types to be differentiated (6). Xylen/chloroform incubation of tissue sections dissolves MMA completely revealing more antigenicity and morphological details for better immunohistology (13, 18). For Technovit 9100 New®, (Heraeus Kulzer, Wehrheim, Germany) a low-temperature acrylic resin embedding method can be favoured over paraffin embedding.

Key words: immunohistochemistry, decalcified bone, non-decalcified bone, plastic-embedding, paraffin-embedding, osteocalcin, osteonectin, osteopontin, collagen type I, cellular marker CD34, cellular marker CD68, human bone

IMMUNOHISTOCHEMICAL COMPARISON OF DIFFERENTIATION MARKERS ON PARAFFIN AND PLASTIC EMBEDDED HUMAN BONE SAMPLES

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To assess bone pathologies and bone regeneration immunohistochemistry may provide additional information compared to conventional histology. However, the effectiveness of this technique is limited due to tissue fixation, preparation and embedding. For bone tissue the standard immunohistological procedure includes formalin fixation, followed by decalcification and paraffin embedding. This may lead to a badly preserved trabecular bone structure but allows antibody application. Alternatively, methyl-methacrylate (MMA) resin may be used for embedding, thus circumventing the decalcification procedure. In this study immunohistology of typical bone markers was compared using human bone samples fixed either with alcohol or formalin and further decalcified and embedded in paraffin and decalcified or non-decalcified samples embedded in Technovit 9100 New®. On semi-thin sections immunohistochemistry with bone markers osteocalcin, osteonectin, osteopontin, collagen type I and the cellular markers CD34 and CD68 was performed. Independent of the fixative used, Technovit 9100 New® embedded non-decalcified bone yielded a stronger immunostaining for all markers when compared to decalcified bone embedded either in methyl-methacrylate or paraffin. In addition there was a better preservation of the trabecular bone morphology. The immunohistochemical results demonstrate that Technovit 9100 New® as a low-temperature acrylic resin embedding method can be favoured over paraffin embedding.

Key words: immunohistochemistry, decalcified bone, non-decalcified bone, plastic-embedding, paraffin-embedding, osteocalcin, osteonectin, osteopontin, collagen type I, cellular marker CD34, cellular marker CD68, human bone
antigenicity and enzyme activity in non-decalcified bone (20) and other tissues e.g. cytokeratins (21) or TRAP (tartrate-resistant acid phosphatase) (17). However, it is clear that for each antibody the circumstances of fixation and tissue embedding have to be assessed separately. Typical bone matrix markers have been determined earlier following different histological preparation procedures (Table 1).

Presently, no published data exist that compare Technovit 9100 New® embedding with the standard paraffin embedding on bone although antibodies staining on paraffin also showed good results on this MMA resins (16) and first immunohistochemistry and enzyme histochemistry has been done by Yang et al. (19).

In the present study, paraffin and Technovit 9100 New® resin embedding were compared for immunohistochemistry of osteocalcin, osteonectin, osteopontin, collagen type I and CD34, CD68 on non-decalcified and decalcified human trabecular bone biopsies, in order to establish which procedure provided optimal staining results.

**MATERIAL AND METHODS**

**Bone tissue**

Bone biopsies were obtained from 29 patients aged between 18 and 77 years undergoing surgery at the Department for Oral and Maxillofacial Surgery, University Hospital Carl Gustav Carus Dresden, Technical University Dresden. With the patients’ informed consent, samples (size max. 5 x 5 x 5 mm) were taken from the hip, the maxilla or the mandible during dento-alveolar or pre-prosthetic bone surgery (augmentation of the alveolar crest using iliac bone graft according to the regulations of the Ethic Commission of the University Hospital Carl Gustav Carus Dresden).

**Processing**

Fresh bone samples were processed according to the following protocol (Table 2). Fresh bone biopsies were halved; one half was fixed in 70% ethanol at 4°C for 24-48 hours the other in buffered 4% formaldehyde at 4°C for 24-48 hours. Each half was then divided into three parts. Per biopsy, one part of the ethanol fixed and one part of the formalin fixed bone was decalcified by EDTA (Osteosoft, Merck, Darmstadt, Germany) at 37°C for 2 days and rinsed with water. All samples were dehydrated by increasing gradients of 70%, 80%, 96%, 96%, 96%, 100%, 100% and 100% ethanol under vacuum (Histokinette-Leica, Wetzlar, Germany) for 12 hours each step at 4°C. Samples were then degreased in xylene for 12 hours twice at 4°C (to facilitate penetration of the embedding medium) before embedding in either paraffin or Technovit 9100 New® resin.

**Paraffin embedding**

Samples were directly taken from the Histokinette and plugged with hot paraffin at 70°C. Semi-thin sections (3 µm) were produced with a rotation microtome (RM 2155, Leica, Wetzlar, Germany).

**Technovit 9100 New® embedding**

Samples were embedded in Technovit 9100 New® resin (Struers, Denmark) for 24-48 hours at 4°C. Sections (2-3 µm) were cut with a microtome (HM 355, Leica, Wetzlar, Germany) and stained with hematoxylin and eosin (H&E).

**Table 1.** Bone proteins in human adult bone: negative intensity (--), weak staining (+), medium intensity (+++) or strong staining (+++); common immunohistochemical staining results (18, 19, 22).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Osteocalcin</th>
<th>Osteonectin</th>
<th>Osteopontin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoid</td>
<td>+</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>Periosteum</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Osteoblasts</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Osteocytes</td>
<td>+</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>Osteocyte lacunae</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Osteoclasts</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Canalicular</td>
<td>--</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Mineralized bone</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Cement lines</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Mineralization front</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Resorption zone</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

**Table 2.** Processing of human bone samples prior to immunohistochemistry. Two different fixation conditions, decalcification and two embedding procedures exhibit to sets of Pdeca, Tdeca and Tnon-deca, which will be referred to as one in the following.

**Table 3.** List of antibodies used for immunohistochemical stainings.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Clone</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteocalcin</td>
<td>polyclonal</td>
<td></td>
<td>Biotrend Chemikalien GmbH, Köln, Germany</td>
</tr>
<tr>
<td>Osteonectin</td>
<td>IgG1</td>
<td>15G12</td>
<td>Novocastra Laboratories Ltd., Newcastle, UK</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>IgG1</td>
<td>OP3N</td>
<td>Novocastra Laboratories Ltd., Newcastle, UK</td>
</tr>
<tr>
<td>Collagen type 1</td>
<td>IgG1</td>
<td>COL-1</td>
<td>Sigma, St. Louis, USA</td>
</tr>
<tr>
<td>CD34</td>
<td>IgG1</td>
<td>B1-3C5</td>
<td>DakoCytomation, Glostrup, Denmark</td>
</tr>
<tr>
<td>CD68</td>
<td>IgG1</td>
<td>KP1</td>
<td>DakoCytomation, Glostrup, Denmark</td>
</tr>
</tbody>
</table>
Technovit 9100 New® embedding

Samples were directly taken from the Histokinette and embedded in Technovit 9100 New® (Heraeus Kulzer GmbH, Germany). After two pre-infiltration steps with xylene and Technovit 9100 New® ratio 1:1 for 12 hours each, the bone tissue was embedded in Technovit 9100 New® consisting of monomeric methacrylate (PMMA), dibenzoyl peroxide (catalyst 1), N,N-3,5-tetramethyl-aniline (catalyst 2), decane-1-thiol (regulator), and poly-methyl-methacrylate (PMMA) powder. Infiltration and polymerization solutions were prepared according to the manufacturer's recommendations. Polymerization was carried out at -2°C in the refrigerator under vacuum for two days. Semi-thin sections were prepared and methyl-methacrylate was removed using twice xylene for 20 min, twice 2-methoxyethylacetat for 20 min, twice acetone for 5 min, and 80% ethanol. Semi-thin sections (3 µm) were cut (Rotationmikrotom Supercut 2065, Reichert-Jung, Nußloch, Germany) and attached to silanized glass slides. Sections were flattened with 5% ethanol, collected, pressed in a compactor, and kept in an incubator at 60°C for 2 hours. Then sections cooled to room temperature, resin was removed in xylene, 2-methoxyethylacetat, acetone for 5 min, and 80% ethanol. Semi-thin sections (3 µm) were cut (Rotationmikrotom Supercut 2065, Reichert-Jung, Nußloch, Germany) and attached to silanized glass slides. Sections were flattened with 5% ethanol, collected, pressed in a compactor, and kept in an incubator at 60°C for 2 hours. Then sections cooled to room temperature, resin was removed in xylene, 2-methoxyethylacetat, acetone, 80% ethanol and hydrogen peroxide.

Immunohistochemistry

For indirect immunohistochemistry, the epitopes blocked during fixation and embedding were exposed using the following demasking procedure. In brief, after removal of the embedding material, endogenous peroxidase was blocked with H2O2. Then, semi-thin sections were rinsed in saline and incubated in phosphate buffered citrate solution pH 7.4 (Dako, Glostrup, Denmark) for 20 min in the microwave and rinsed with phosphate buffered saline (PBS). Unspecific binding was blocked with 10% horse serum (Vector Laboratories, Burlingame, CA, USA), primary antibodies (as listed in Table 3) were incubated for 8 h at 4°C, afterwards the secondary biotin-conjugated antibody (Vectastain universal Elite ABC-Kit, Vector Laboratories, Burlingame, CA, USA) was incubated for 1 h at room temperature. The avidin-biotin reagent (Vector Laboratories, Burlingame, CA, USA) was applied for 30 min. Sections were counterstained with HistoGreen solution as chromogen substrate (Histoprime®, Linaris, Wertheim-Bettingen, Germany) and hematoxylin (Merck, Darmstadt, Germany) for 10 seconds. Between incubation steps saline rinses were performed. The sections were mounted in 40% glycerol gelatine (Merck, Darmstadt, Germany) in PBS. As control, the staining procedures were carried out without primary antibody or with non-specific immunoglobulins of similar isotypes as the primary antibodies (IgG1, IgG2a, IgG2b, IgM; ICN, Meckenheim Germany). All antibodies were diluted according to the manufacturers instructions.

Histomorphometry and statistics

The sections were evaluated with a light microscope (Olympus BX-61, Hamburg, Germany) using an integrated digital CCD-camera ColorView II and digital image acquisition and analyzing system (CellF AnalySIS, Soft Imaging Systems, Munster, Germany). Analysis consisted of a morphologic description and an evaluation for each antibody of the positively stained areas (in µm²) in relation to the total bone surface per section (in µm²) in percentage. The data analysis was performed by SAS procedure LOGISTIC and MIXED using a linear model of covariance analysis with the parameters: a) 3 locations (hip, maxilla, mandible), b) 2 fixatives (formalin, ethanol), c) 2 genders as binary co-variable and age (a-c data not shown) and d) 2 embedding media (paraffin and Technovit 9100 New®) as general co-variable.

![Graphs showing statistical evaluation of the influence of embedding on the immunohistochemical staining result. BoxPlots A show values of Pdeca, BoxPlots B show values of Tdeca, BoxPlots C show values of Tnon-deca. Depicted statistics for osteocalcin (OCN), osteonectin (ONC), osteopontin (OPN), Collagen I and CD68 are significant (p=0.005). For CD34 the values were not statistically significant.](image-url)
These parameters required an analysis of high complexity including repeat experiments on samples from the same patient. Therefore compound-symmetry-covariance structure was applied, regarding all intra-individual correlations as identical. Experimental data were factorized into two target variables: I) binary reaction parameter (yes/no); II) Intensity of reaction, if >0 with the advantage to circumvent the non-parametrical analysis. Factorization was based on the assumption that the magnitude of reaction is approximately normal and was analysed in a linear mode. For the binary reaction parameter a logistic regression model was used. To calculate the reaction intensity a linear mode of covariance analysis for the quotient logarithms was arranged. Taking the logarithms with respect to the Box-Cox-transformation generates an approximated normal distribution (box plots). Arithmetic means were Tuckey-adjusted afterwards. Calculated values of arithmetic means, variance in between the measurements and standard deviation were tested in Kolmogorov-Smirnov-Z test for normal distribution and for statistical significance respectively. The critical region of null hypothesis was set to 5%. This two-dimensional analysis needs to check probability distributions from both sides with significance levels of 2.5%. To check the variables "location", "fixative", "embedding medium", "age" and "gender" for interaction, data were applied to a multivariate variance analysis. The critical region was set to 5% again (Fig. 1 for embedding medium). There were no significant influences on the immunohistological results by other parameters like origin of the bone biopsy, gender or age of the patient (data not shown).

RESULTS

Osteocalcin

The osteocalcin (OCN) antibody stained the areas of osteoid, the seam of osteoblasts in the mineralized bone, the osteocyt lacuna and the cement lines. The methyl-methacrylate sections of non-decalcified (Tnon-deca) bone revealed a stronger positive reaction in the localization mentioned. Compared to decalcified plastic (Tdeca) or paraffin (Pdeca) embedded bone the difference was statistically highly significant (Fig. 1, Fig. 2A-C).

Osteonectin

There was a positive staining for osteonectin (ONC) around osteoblasts and osteocytes in areas of osteoid, of mineralized bone and of bone resorption. In methyl-methacrylate sections

![Fig. 2. Osteocalcin (OCN) staining of osteoid and cementlines in Pdeca (A), Tdeca (B), around osteons and osteocyte lacunae in Tnon-deca (C). Osteonectin (ONC) staining was observed in osteoblastic seam around osteons in Pdeca (D), osteoid Tdeca (E) and enclosed osteocytes Tnon-deca (F). Osteopontin (OPN) was found in the same locations in Pdeca (G), Tdeca (H) and Tnon-deca (J). Collagen type I was observed in osteoid Pdeca (K) and enclosed osteocytes Tdeca (L) and in both Tnon-deca (M).](image)
there was a positive staining along the basement membranes of blood vessels. The reactivity for osteonectin in non-decalcified (Tnon-deca) bone was stronger. Compared to decalcified plastic (Tdeca) or paraffin (Pdeca) embedded bone the difference was statistically highly significant (Fig. 1, Fig. 2D-F). In addition there was also a stronger reaction in samples fixed with ethanol.

**Osteopontin**

The glycoprotein osteopontin (OPN) was localized around the osteocyte lacunae, in the osteoid along the cement lines and in mineralized bone. There was a significantly stronger reaction in respect to formalin fixed tissue, and to non-decalcified methyl-methacrylate embedded tissue (Tnon-deca) (Fig.1, Fig. 2G-J). In addition male bone tissue expressed more osteopontin (data not shown).

**Collagen type 1**

A positive immunostaining for collagen type 1 was found around osteoblasts, in the osteoid, in resorption areas, in cement lines and in the mineralized matrix. In regard to occurrence the immunoreaction was significantly more prominent after ethanol fixation compared to formalin. In non-decalcified methyl-methacrylate embedded bone (Tnon-deca) there was significantly stronger staining (Fig. 1, Fig. 2K-M). Furthermore, the expression of collagen type 1 was reduced per factor 0.019 per year of age.

**CD 34**

The marker for hematopoietic stem cells and capillary endothelial cells provided a staining around vessels which was stronger in formalin fixed bone samples of methyl-methacrylate embedded bone (Tnon-deca) (Fig. 1, Fig. 3A-C). Male bone tissue expressed significantly more CD34 (data not shown).

**CD 68**

The marker for macrophages located in peripheral tissues macrophages, monocytes and granulocytes. In non-decalcified as well as in decalcified bone tissue embedded in Technovit 9100 New® osteoclasts showed a stronger positive staining signal than in paraffin embedded bone tissue (Fig. 1, Fig. 3D-F). A stronger reaction was observed in samples fixed with formalin.

**DISCUSSION**

Collagenous and non-collagenous matrix proteins play an important role in the architecture and function of bone. To study their proper localization using immunohistochemical techniques is interesting for different areas of research. The developing presumptive bone tissue is an interesting topic for us as we have been focusing on regenerative approaches for curing bone deficiencies and clinically relevant defects using tissue engineering. A major drawback is that bone as hard tissue is difficult to reach for routine immunohistological techniques. Modifications of tissue preparations and embedding techniques should be considered to benefit laboratory results. As we have recently assessed the immunostaining of soft tissue markers in MMAembedded material (21), the focus of this study was to develop and optimize the approach to localize and morphometrically quantify specific bone markers. Using different fixation and embedding techniques the immunostaining of different bone specific markers was compared. Advantages arising from the embedding of mineralized bone in MMA instead of paraffin include better preserved morphology and a more precise assignment of protein and bone mineral (13, 18, 19).

Disadvantages of MMA embedding already have been reported: due to oxidative and other chemical reactions of MMA monomer with parts of the cytoplasm, which results in an increased affinity of basic dyes, there is a tendency to a prevailing blue staining (6). This blue staining was not observed using Technovit 9100 New®. Furthermore, the tissue shrinkage and cracks described by Yang et al. (19) using the same embedding material were hardly observed. In general, bone morphology was sufficient, although cracks and folds were observed in particular in demineralized tissue independent of the embedding material.

Decalcified material showed a significantly reduced immunohistochemical reaction. Especially strong mineralic acids are known to hinder antigenicity, distort tissue morphology and reduce the quality of staining results (23).
To improve the quality of the histological results an optimal dilution of the primary antibody is required. In contrast to the manufacturer's recommendation, higher dilutions of the antibodies have been used. An increase in sensitivity was achieved using the Avidin-Biotin-method (24) and the demasking of the antigens using citrate phosphate buffer in the microwave (25).

Osteocalcin, the most bone specific non-collagenous matrix protein, was localized beside osteoblasts, osteoid and the mineralized bone also in the osteocyte lacunes and the cement lines. These findings are in accordance with those of Derkx et al. (18). Osteocalcin is specific also for both osteoblasts and osteocytes (26).

Osteonectin is not only produced by osteoblasts but also by other cells, thus explaining its localization along the basal membrane of blood vessels. This localization was found in calcified plastic embedded sections only. Otherwise, the osteonectin localization around osteoblasts, osteocytes, in the osteoid, in mineralized bone and in resorption areas is in agreement with previous descriptions (18, 19).

Osteopontin immunostaining in osteocyte lacunae, osteoid, cement lines and in mineralized bone confirm previous studies (19, 22). In addition, as described by Liaw et al. (27), there was immunostaining of osteopontin along the outer zones of the skeletal muscles. Regular immunolocalization of osteopontin seems to be critically dependent on antigen retrieval (28).

Collagen type I was found around osteoblasts, cement lines, osteoid, in the mineralized matrix and in resorption areas. These findings are in agreement with those of Yang et al. (19). In addition, there was an age related decrease of the collagen content in the bone which has been previously described by Chen et al. (29), which may subsequently lead to reduced elasticity of the bone.

Immunohistochemical stainings of CD34 showed protein localizations around small vessels and capillary structures of the bone samples independent of embedding or fixation parameters. This observation was achieved following recommendations of Schaefer (6), who suggested the use of CD34 antibody in combination with the ABC-method to characterize bone biopsies immunohistochemically.

The macrophage marker CD68 showed protein stainings located not only in macrophages but also granulocytes, myeloid precursors and osteoclasts emerged from cellular fusion of monocytes as previously reported by Athanasou et al. (30).

Based on the immunohistochemical results and the deduced statistical calculations presented above, the analyses of MMA based Technovit 9100 New® embedded non-decalcified bone samples have shown to be significantly predominant over all other tested histological preparation techniques. It was concluded that due to the decalcification procedure, antigenicity of the bone samples was severely diminished. Technovit 9100 New® embedding allowed in all cases a stronger antigen-antibody binding, except for the staining result of CD34 which was independent of the embedding. Comparing the fixatives formalin and ethanol, there were no striking histomorphological differences observed. Formalin fixation preserved a better antigen structure as shown for osteopontin, CD34 and CD68, whereas ethanol maintained a better antigenicity of osteonectin and collagen type 1.

Acknowledgements: The work was supported by a seed grant "Establishing optimized techniques for cell tracking in controlled in situ bone regeneration" of the DFG Research Center for Regenerative Therapies Dresden (CRTD). The skilled technical assistances of Ines Kleiber and Diana Jünger are highly appreciated.

Conflict of interests: None declared.

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Received: October 22, 2009
Accepted: December 18, 2009

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