J Bone Miner Metab (2008) 26:110–113
DOI 10.1007/s00774-007-0788-2

RAPID COMMUNICATION

**Samirah Abreu Gomes · Luciene Machado dos Reis
Ivone Braga de Oliveira · Irene de Lourdes Noronha
Vanda Jorgetti · Ita Pfeferman Heilberg**

Usefulness of a quick decalcification of bone sections embedded in methyl metacrylate: an improved method for immunohistochemistry

Received: March 26, 2007 / Accepted: June 29, 2007

Abstract Immunohistochemistry of undecalcified bone sections embedded in methyl methacrylate (MMA) is not commonly employed because of potential destruction of tissue antigenicity by highly exothermic polymerization. The aim of the present study was to describe a new technique in which a quick decalcification of bone sections embedded in MMA improves the results for immunohistochemistry. The quality of interleukin 1α (IL-1α) immunostaining according to the present method was better than the conventional one. Immunostaining for osteoprotegerin (OPG) and the receptor activator of NF-κB ligand (RANKL) in bone sections of chronic kidney disease patients with mineral bone disorders (CKD-MBD) was stronger than in controls (postmortem healthy subjects). The present study suggested that this method is easy, fast, and effective to perform both histomorphometry and immunohistochemistry in the same bone fragment, yielding new insights into pathophysiological aspects and therapeutic approaches in bone disease.

Key words bone · immunohistochemistry · methyl methacrylate · osteoprotegerin · chronic kidney disease

Introduction

Plastic embedding, especially with methyl methacrylate (MMA), provides many advantages for bone histology analysis. In this procedure, the preservation of structural details is usually excellent. However, it leads to severe chemical alterations of the tissue and thereby prevents the successful application of immunohistochemistry [1]. It has been recognized for a long time that minimizing polymerization temperatures during the MMA embedding process is critical for preserving the tissue enzyme activity and antigenicity [2]. In 1992, Wolf et al. [3] described a MMA technique involving such procedures. However, it involved tedious purification and destabilization of the methacrylate components. In 1997, a study obviated the need of this purification [4]. A subsequent report by Yang et al. [5] has shown antigenic preservation in undecalcified trabecular bone following embedding with Technovit 9100 New. In a previous study [6], we were able to show, by means of immunohistochemistry in undecalcified MMA bone sections, the expression of cytokines such as interleukin (IL)-1β, tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), and basic fibroblast growth receptor (bFGF) in iliac crest bone biopsies from chronic kidney disease patients with mineral bone disorders (CKD-MBD patients). In the present investigation, we aimed to demonstrate that a quick decalcification of bone sections embedded in MMA improves the results for immunohistochemistry.

S.A. Gomes (✉) · I.P. Heilberg
Division of Nephrology, Universidade Federal de São Paulo, Rua Botucatu, 740 Vila Clementino, São Paulo, SP Brazil 04023-062
Tel. +55-11-5574-6300; Fax +55-11-5573-9652
e-mail: samirah@nefro.epm.br

L.M. dos Reis · I.B. de Oliveira · I.L. Noronha · V. Jorgetti
Nephrology Division,Universidade de São Paulo, São Paulo, Brazil

Patients and methods

Undecalcified bone fragments obtained from 10 iliac crest bone biopsies previously performed in CKD-MBD patients and 10 postmortem bone samples from healthy subjects, used as controls, were included. This study was approved by the Ethical Committee of the Federal and State Universities of São Paulo, and all patients had signed a written consent by the time those bone biopsies had been performed.

Interleukin 1α was used to show the comparison between the conventional and the present method. Osteoprotegerin and RANKL were chosen for testing immunostaining because of their abundance in bone tissue and importance as key regulators of bone remodeling.

Bone sections were deacrylated in a 1 : 1 mixture of xylene and chloroform for 30 min, rehydrated in graded alcohol solutions, submitted to a quick decalcification with 1% acetic acid for 10 min, rinsed twice with distilled water, and treated with 0.1% Tween 20 in phosphate-buffered saline (PBS). Endogenous peroxidase activity was inhibited by a mixture

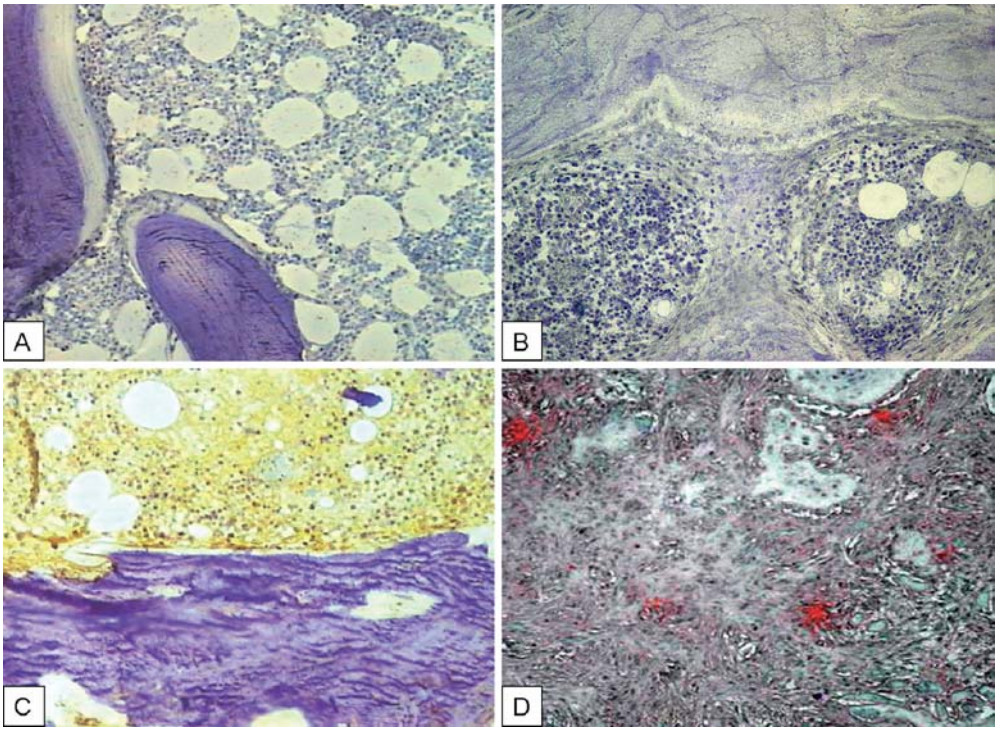

Fig. 1. Comparison of immunohistochemical reaction in a negative control (omitting the primary antibody) revealing no labeling using the conventional method (**A**) or the present method, showing no background (**B**). IL-1α immunostaining in a chronic kidney disease patient with mineral bone disorders (CKD-MBD) performed by the conventional method presented marked labeling (*brown*) with a slight background (**C**), and marked labeling (*red*) through the new method (**D**), with no background and better quality

of 3% hydrogen peroxide in methanol for 30 min, followed by two water washes. The samples were incubated with avidin-biotin solutions and with 5% normal serum from the same species of secondary antibody with 1% bovine serum albumin (BSA) to block nonspecific bindings. Sections were incubated overnight at 4°C in a humidified chamber using primary goat monoclonal antibody antihuman osteoprotegerin (OPG) (dilution 1:80) and goat monoclonal antibody antihuman RANKL (1:150) in 1% bovine serum albumin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with the primary antibody, sections were rinsed in PBS, incubated with matching biotinylated secondary antibodies, and blocked with 5% human serum. The slides were then incubated with streptavidin ABC complex/HRP (ABC, 1:300; ABC kit; Vector, Burlingame, CA, USA) for 30 min. Antigen–antibody complexes were visualized using a 3-amino-9-ethylcarbazole substrate chromogen (AEC) (Sigma Chemical, St. Louis, MO, USA). The sections were rinsed in distilled water and counterstained with Mayer's hemalum solution (Merck, Darmstadt, Germany). Simultaneous negative controls were carried out by omitting primary antibody in all sections.

Quantitative expression of OPG and RANKL in bone biopsies was measured by the point-counting technique [6] at a magnification of 100× and using a 176-point ocular grid. Counting was performed on 25 microscopic fields for each bone sample. Each point was counted as either "positive" or "negative." The area of immunopositivity in the tissue was determined by the number of positive points in the tissue compared to the total number of points. The results are expressed as a percentage of total tissue. Unpaired *t* test was used to compare results from both groups. The level of significance was set at $P < 0.05$.

Results

Figure 1 shows the comparison between the conventional immunohistochemical technique [6] and that performed in the present study utilizing IL-1α in bone tissue of a CKD-MBD patient. Absence of immunostaining and no background in the negative control (omitting the primary antibody) with the present technique (Fig. 1B) compared with the conventional method (Fig. 1A) were observed. IL-1α bone immunostaining with the new technique (Fig. 1D) also presented better quality than the conventional method (Fig. 1C). OPG and RANKL bone expression in CKD-MBD patients is shown in osteoblasts next to the osteoid surface, in some medullary cells, in mature osteocytes, in Fig. 2. Toluidine blue staining is shown to evidence osteoblast localization (Fig. 2A,D), and higher reactivity for both OPG (Fig. 2B) and RANKL (Fig. 2E) was observed in a CKD-MBD patient when compared with a healthy subject for OPG (Fig. 2C) and RANKL (Fig. 2F), respectively. Mean values of OPG and RANKL bone immunostaining in CKD-MBD patients were significantly higher than in healthy subjects (3.6 ± 2.1 vs. 0.1 ± 0.1 and 3.7 ± 2.0 vs. $0.2 \pm 0.1\%$, $P < 0.0005$).

Discussion

Methyl methacrylate embedding allows a distinction between mineralized bone and unmineralized osteoid, with an excellent preservation of the bone and cellular structures, which are mandatory for a reliable bone histomorphometric analysis. However, it interferes with immu-

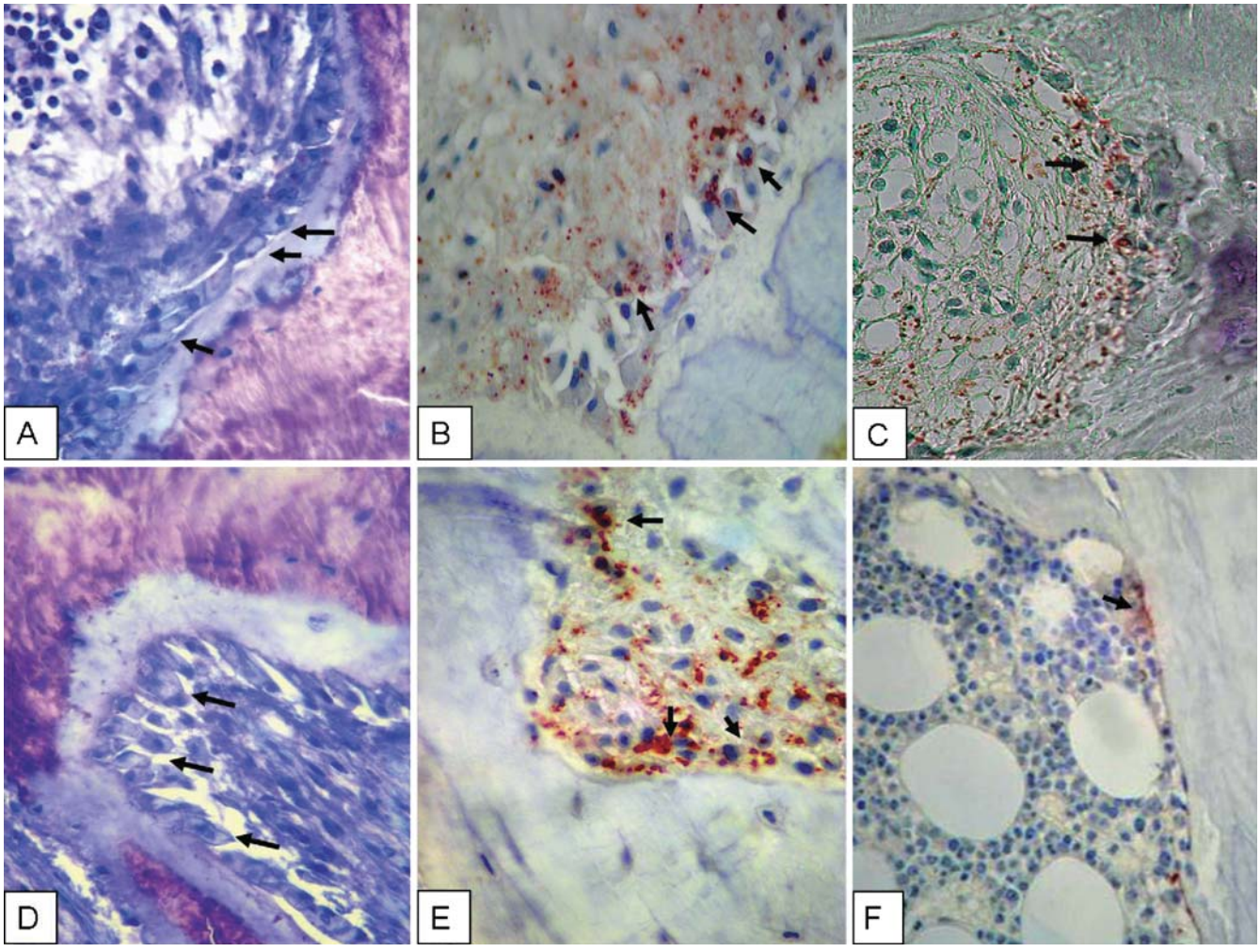

Fig. 2. Osteoblasts are stained with toluidine blue (*arrows*) in a CKD-MBD patient (**A**); osteoblasts along the trabecular borders and medullary cells (*arrows*) are markedly labeled in *red* for osteoprotegerin (OPG) in this CKD-MBD patient (**B**) and less stained in a healthy subject (**C**). Bone sections labeled for RANKL were also tested with toluidine blue to show osteoblasts localization (**D**); marked immunostaining for RANKL is also shown in a CKD-MBD patient (**E**) and weaker expression in a healthy subject (**F**). ×400

nohistochemistry performance unless high temperatures for MMA polymerization are avoided [2–6]. The combination of histomorphometry and immunohistochemistry in the same bone fragment may be crucial for better pathophysiological insights.

We report here a new technique using standard MMA embedding that enabled us to perform both morphological and immunohistochemical analysis in the same bone fragment with optimal results of the latter. Although several studies have already described both analyses being performed together with good results [3,4,6,7], they took almost 3 days to complete. The present study provided reliable data in a shorter time (1.5 days) and with much better quality of immunostaining visualization with no background. The main factor that contributed for this timing was the utilization of a mixture of xylene and chloroform only for 30 min with a optimal deacrylation, as suggested by Derkx et al. [8], whereas other studies [6,7] have utilized acetone for at least 12 h, posing the risk of causing tissue damage and fixation losses of bone section. Another crucial point that helped ameliorating the quality of the immunostaining in our study was a quick decalcification with acetic acid for 10 min, removing the mineralized bone matrix for better exposure of epitopes and the utilization of Tween 20, a nonionic detergent, a solubilizing and blocking agent for membrane, before endogenous peroxidase blockage.

Given the role of OPG and RANKL in controlling osteoclastogenesis, we selected CKD-MBD patients to evaluate OPG and RANKL immunostaining. This is the first human study to report strong OPG and RANKL bone immunostaining in CKD-MBD patients. The OPG and RANKL bone immunolocalization detected in our study matches with other reports of OPG localization by Northern blot analysis in bone cells [9–11]. In experimental studies, performed in paraffin-embedded bone tissue [12,13], a similar localization of OPG and RANKL has also been detected. Because the present study was performed in bone tissue retrieved from previous biopsies, Western blot analysis could not be carried out because the specimen had been submitted to fixation.

In conclusion, the present study suggested that this novel technique is an easy, fast, and effective method that can be routinely used for both histomorphometry and immunohistochemistry, helping to bring better comprehension of the physiopathogenic mechanisms in various bone diseases. The possibility of detecting immunostaining for bone proteins such as OPG and RANKL and other cytokines in human iliac crest bone biopsies previously submitted to histomorphometry may be an additional advantage.

Acknowledgments This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). The authors thank Rosimeire Aparecida Bizerra da Costa, Rita Martin, and Wagner Vasques Dominguez of Renal Pathophysiology Laboratory of Universidade de São Paulo, for technical assistance.

References

1. Merrel GA (2005) Effects of long-term fixation on histological quality of undecalcified murine bones embedded in methyl methacrylate. Biotech Histochem 80:139–146
2. Ruddell CL (1967) Hydroxyethyl methacrylate combined with polyethylene glycol 400 and water; and embedding medium for routine 1–2 micron sectioning. Stain Technol 42:119–123
3. Wolf E, Roser K, Hahn M, Welkerling H, Delling G (1992) Enzyme and immunohistochemistry on undecalcified bone and bone marrow biopsies after embedding in plastic: a new embedding method for routine application. Virchows Arch A Pathol Anat Histopathol 420:17–24
4. Erben RG (1997) Embedding of bone samples in methyl methacrylate: an improved method suitable for bone histomorphometry, histochemistry, and immunohistochemistry. J Histochem Cytochem 45:307–313
5. Yang R, Davies CM, Archer CW, Richards RG (2003) Immunohistochemistry of matrix markers in Technovit 9100 New-embedded undecalcified bone sections. Eur Cell Mater 6:57–71
6. Santos FR, Moyses RM, Montenegro FL, Jorgetti V, Noronha IL (2003) IL-1beta, TNF-alpha, TGF-beta, and bFGF expression in bone biopsies before and after parathyroidectomy. Kidney Int 63:899–907
7. Duarte ME, Carvalho EF, Cruz EA, Lucena SB, Andress DL (2002) Cytokine accumulation in osteitis fibrosa of renal osteodystrophy. Braz J Med Biol Res 35:25–29
8. Derkx P, Nigg AL, Bosman FT, Birkenhager-Frenkel DH, Houtsmuller AB, Pols HA, van Leeuwen JP (1998) Immunolocalization and quantification of noncollagenous bone matrix proteins in methyl methacrylate-embedded adult human bone in combination with histomorphometry. Bone (NY) 22:367–373
9. Udagawa N, Takahashi N, Jimi E, Matsuzaki K, Tsurukai T, Itoh K, Nakagawa N, Yasuda H, Goto M, Tsuda E, Higashio K, Gillespie MT, Martin TJ, Suda T (1999) Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor: receptor activator of NF-kappa B ligand. Bone (NY) 25:517–523
10. Kartsogiannis V, Zhou H, Horwood NJ, Thomas RJ, Hards DK, Quinn JM, Niforas P, Ng KW, Martin TJ, Gillespie MT (1999) Localization of RANKL (receptor activator of NF kappa B ligand) mRNA and protein in skeletal and extraskeletal tissues. Bone (NY) 25:525–534
11. Zhao S, Zhang YK, Harris S, Ahuja SS, Bonewald LF (2002) MLO-Y4 osteocyte-like cells support osteoclast formation and activation. J Bone Miner Res 17:2068–2079
12. Nakamura H, Tsuji T, Hirata A, Yamamoto T (2002) Localization of osteoprotegerin (OPG) on bone surfaces and cement lines in rat tibia. J Histochem Cytochem 50:945–953
13. Silvestrini G, Ballanti P, Patacchioli F, Leopizzi M, Gualtieri N, Monnazzi P, Tremante E, Sardella D, Bonucci E (2005) Detection of osteoprotegerin (OPG) and its ligand (RANKL) mRNA and protein in femur and tibia of the rat. J Mol Histol 36:59–67