

**Osteopontin is an argentophilic protein in the bone matrix and in
cells of kidney convoluted tubules**

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ABSTRACT

Nucleolar Organising Regions (NOR) are part of the nucleolus, containing argyrophilic proteins (nucleolin/C23, nucleophosmin/B23). They are identified by silver staining at low pH. The method also reveals osteocyte canaliculi and cement lines and granules in the cytoplasm of kidney cells in locations that mimic osteopontin distribution. Human bone and kidney sections, benign and lymphomatous pleural effusions were processed for silver staining to identify AgNOR. Sections were processed in parallel for immunohistochemistry with an antibody direct against osteopontin. In pleural effusions, AgNORs were found increased in the nuclei of lymphoma cells. In bone, Ag staining identified AgNOR in cell nuclei, as well as in osteocyte canaliculi, cement and resting lines. In the distal convoluted tubules of the kidney, silver deposits were also observed in cytoplasmic granules on the apical side of the cells. Immunolocalization of osteopontin closely matched with all these locations in bone and kidney. NOR proteins and osteopontin are proteins containing aspartic acid rich repeats that can bind Ag. Staining protocols using silver nitrate at low pH can identify these proteins on histological sections. AgNOR is a useful histochemical method to identify osteopontin in bone sections.

Short running title: Osteopontin and silver staining

Key words: Osteopontin; AgNOR Proteins; Silver Staining; Histochemistry; Non Collagenous Proteins.

RESUME

Les régions organisatrices du nucléole (NOR) constituent une fraction nucléolaire, contenant des protéines argyrophiles (nucléoline/C23, nucléophosmine/B23). Elles sont identifiées histochimiquement par coloration argentique à pH acide. La méthode détecte également les canalicules des osteocytes, les parois des ostéoplastes, les lignes cimentantes osseuses. Ces localisations évoquent la distribution tissulaire de l'ostéopontine. Des liquides pleuraux bénins et lymphomateux, des coupes d'os et de rein (autre tissu contenant de l'ostéopontine), ont été traitées par une coloration argentique identifiant les NORs (AgNOR). Des coupes ont été traitées en parallèle par immunohistochimie avec un anticorps dirigé contre l'ostéopontine. Dans liquides pleuraux, les AgNORs ont été trouvées en nombre élevé dans les noyaux des cellules lymphomateuses. Dans l'os, la coloration a identifié les AgNOR dans les noyaux mais elle est aussi positive dans des canalicules des osteocytes et les lignes cimentantes. Dans les tubules contournés distaux du rein, on a également observé des dépôts granulaires d'argent au pôle apical des cellules. L'immunolocalisation de l'ostéopontine était similaire, dans l'os et le rein, aux zones marquées par la méthode AgNOR. Les protéines nucléolaires et l'ostéopontine sont des protéines contenant les séquences riches en acide aspartique qui peuvent fixer les ions Ag. Ces protocoles AgNOR employant le nitrate d'argent à pH faible représentent une méthode histochimique utile pour identifier l'ostéopontine.

Titre court : Ostéopontine et coloration argentique

Mots clés : Ostéopontine, Protéines AgNOR, coloration argentique, histochimie, protéines non-collagéniques.

INTRODUCTION

Bone matrix is composed of both a mineral phase associated with an organic phase deposited and textured by osteoblasts. Collagen type I is the main component of the organic phase and confers elasticity to the tissue while the mineral phase is responsible for its rigidity and strength. Although collagen account for more than 90% of the organic phase, more than 200 other proteins have been identified by biochemical analysis and their respective roles are either partially elucidated or still completely unknown at that time. Several proteins are synthesized by osteoblasts and embedded in the matrix at the time of osteoid deposition (e.g., alkaline phosphatase, bone sialoprotein -BSP, osteocalcine, bone morphogenetic proteins...)[1, 2]. Numerous other proteins are elaborated elsewhere in the body and are passively deposited into the matrix (e.g., α 2-HS glycoprotein from the liver, fibronectin, thrombospondin...)[3]. Several non collagenic proteins possess acidic aminoacid residues that allow them to anchor on the hydroxyapatite crystals of the mineral phase: osteocalcin possesses three carboxylic residues [4], BSP contains several glutamic acid repeats and osteopontin contains several aspartic acid sequences. We have previously found that a technique developed to stain the Nucleolar Organizer Regions (NORs) using silver nitrate in an acidic buffer was also working on bone sections [5]. The AgNOR method was found to be effective in labeling NORs in bone cell nuclei but also revealed the canaliculi and lacuna margins of osteocytes together with the cement lines of the bone structure units. Because this location was similar to that of osteopontin, we investigated the possibility for the AgNOR staining method to be a suitable histochemical technique for osteopontin. The present paper summarizes the potent interest of AgNOR staining in bone biology.

MATERIAL AND METHODS

Patients

Three femoral heads, obtained from patients with hip fractures or arthritis, and subjected to hip prosthesis, were used as control. Human renal cortex was obtained from the normal remaining parenchyma of a patient with cancer of the upper pole. Tissue samples of both kidney and hips were received unfixed soon after surgical

removal. They were immediately sampled with a scalpel or a banding saw. Pleural fluids from ten patients with a benign effusion were compared to ten fluids from patients with a malignant lymphoma. Fluids were cytocentrifuged on a Shandon cytospin, slides were stained by May-Grünwald-Giemsa (MGG) for routine diagnosis, additional smears were stained by the AgNOR technique.

Tissue samples were immersed in freshly prepared 4% paraformaldehyde in sodium phosphate buffer (PBS), pH 7.4, for 24h at +4°C. Bones were decalcified in 0.5M EDTA pH 7.5 at room temperature (RT) for 2weeks, extensively rinsed in PBS, dehydrated in ethanol and embedded in paraffin. Sections (5µm thick) were prepared and mounted on silanized glass slides.

AgNOR staining

Cell smears, bone and kidney sections were incubated for 55min at RT in the dark, in a staining solution prepared by combining silver nitrate (2 volumes of a 50% aqueous solution) with formic acid (1 volume of a 1% solution containing 2% gelatin) [6]. After staining, slides were thoroughly washed in distilled water (DW) and transferred for 10min to a 5% aqueous sodium thiosulfate solution prepared extemporaneously. Sections were then rinsed with DW, mounted and observed by light microscopy. Cytological smears were counterstained with nuclear fast red, dehydrated in ethanol and mounted in Entellan.

Immunohistochemistry of kidney and bone tissue sections

For antigen unmasking, deparaffinized sections were incubated with 0.05% pronase in PBS for 20min at +37°C. The enzymatic reaction was stopped in cold PBS. Saturation of non-specific binding sites was done with 1% bovine serum albumin (BSA) in PBS for 15min. Sections were then incubated overnight at +4°C with a 1:50 rabbit anti human osteopontin peptide (75-90) a primary polyclonal antibody (Immundiagnostik AG, Bensheim, Germany). After rinsing in PBS, immunoreaction was revealed with a 1:100 diluted goat anti-rabbit IgG secondary antibody coupled with Alexa Fluor 488 (Molecular Probes, Eugen, Oregon, USA) for 1h at room temperature. After incubation, sections were extensively rinsed in PBS and mounted in glycerol-PBS (9:1). The immunohistochemical labeling was then visualized by epifluorescence. The specificity

of each antibody has been documented by immunocytochemical controls, including incubation of sections with goat anti-rabbit fluorochrome conjugate.

RESULTS

AgNOR technique on cytological smears and sections.

The histochemical identification of NORs was clearly obtained. In benign pleural effusions, NORs appeared usually as a single dot in the lymphocyte nuclei. When lymphoma cells were present, (characterized cytologically on the MGG stained smears), they exhibited an increased number of NORs (Figure 1).

On bone sections, NORs appear as black dots in nuclei of marrow and bone cells. In osteoclast nuclei, two or three dots were identified. Osteoblasts contained usually one or two AgNORs. Silver deposits were also found associated with osteocytes canaliculi, cement and resting lines within the bone matrix (Figure 2). In such a way, the delicate inter osteocyte relationships were clearly evidenced. Osteoid seams were never stained by the AgNOR technique.

On kidney sections, similar NORs identification was also evidenced in the nucleoli of kidney cells. However, silver precipitates were also seen as small black dots in the cytoplasm of some cells of the cortex. AgNOR staining identified intracytoplasmic granules only in cells of the distal tubule and the ascending limb of the loop of Henle. The black granules were seen in the supra-nuclear area of the cells, toward the lumen of the renal tubules (Figure 3).

Immunolocalization of OPN

Immunohistochemistry revealed a strong and specific signal for osteopontin in the extracellular matrix of bone. Positive labeling was localized at the arrest and cement lines and at the bone surface. In addition, OPN was associated with lining cells, osteocyte canaliculi and in the wall of osteocyte's lacunae (Figure 4A). Immunofluorescence showed that the distribution for osteopontin was only in calcified bone and not in the osteoid. In kidney sections, osteopontin was strongly expressed in the epithelial cells of the distal renal tubules (Figure 4B). The most intense staining was seen at the apical cell side toward the tubule lumen, with lesser fluorescence at the baso-lateral sides.

DISCUSSION

In the nucleolus, DNA ribosomal genes are transcribed into rRNA by RNA polymerase I (Pol I) and RNA topoisomerase I. Ribosomal genes exist in numerous replicated copies (ranging from a few to a thousand) disposed in tandem along the DNA molecule. [7] These genes are separated by segments of nonreplicating DNA (DNA spacer). The fibrillar component of the nucleoli are rounded structures ranging from 100 nm to 1 μ m in diameter and containing fibrils of DNA. The ribosomal genes are transcribed in the dense fibrillar component by a special folding of the DNA molecules that makes all the DNA spacers within the fibrillar center, and the loops of rDNA genes (i.e., the NORs) within the dense fibrillar component [8]. NOR argyrophilia is related to acidic proteins associated with rRNA transcription sites and known to play a key role in the control of rRNA transcription and processing [9]. Nucleolin, protein B23, RNA polymerase I represent the main argyrophilic NORs proteins observed in cell nucleoli [7, 9-13] At the light microscopy level, AgNOR staining methods identify both the fibrillar centers and the associated dense fibrillar component as black dots [6].

Several morphometric studies have stressed the interest of determining the mean AgNOR number per nucleus [8, 9]. An increasing literature has described the use of the AgNORs staining technique to investigate differences in various pathological lesions. Quantitative analysis of interphase AgNORs has been shown to be useful in tumor pathology for distinguishing benign, dysplastic and malignant tumors. Typically, a greater amount of silver dots indicates cells with a high rate of proliferation whether benign (e.g. embryonic) or neoplastic. AgNORs number is correlated with other cell proliferation markers (BrDU, PCNA) [8, 9]. This is clearly shown here where numerous AgNORs are found in the nucleoli of lymphoma cells in pleural effusions. However, in a previous study conducted on bones from patients suffering from Paget's disease, we found an increased number of NORs in the nucleoli of the osteoclasts (6.80 ± 2.57 dots vs. 2.12 ± 1.07 in controls) [14]. Because osteoclasts are formed from fusion of hematopoietic precursors and never by endomitosis, the large number of AgNORs in their nuclei does not reflect the proliferative activity of the cells. The Pagetic osteoclast being highly active, the cytoplasmic synthesis of proteins is maximized (probably hydrolases involved in the matrix breakdown). It is possible that other mRNA (of viral / oncogene origin) [15] could be actively transcribed in pagetic patients and require more

numerous ribosomes or that an oncogene promote alteration of the nuclear/ nucleolar machinery.

AgNOR staining also identified osteocyte canaliculi, cement and resting lines of the bone matrix and granules in the cytoplasm of the renal tubular cells. In the present study, when comparing the two staining methods, AgNOR staining and osteopontin immunohistochemistry, they were found closely correlated in both renal cortex and bone tissues. The resulting OPN immunolabeling reproduced the staining pattern of NOR-silver staining. The relationship between these staining methods was clearly evidenced, since fluorescence and silver precipitate distributions demonstrated a similar localization of OPN expression and argyrophilic sites in human kidney and bone. However, OPN immunolabeling never recognized the NOR in the nucleoli. In previous studies, we also found by transmission electron microscopy that AgNOR could also be used to delineate the osteocyte canaliculi and in the wall of their lacuna [5]. Western blot analysis were also done and immunolabelling and AgNOR staining of gels were similar [16]. OPN is one of the most abundant non-collagenous proteins in bone but its exact roles are not fully elucidated. In vitro studies by several groups have established that OPN is an inhibitor of calcification [17, 18]. Bones from OPN-knockout mice have a greater mineral content and contain larger hydroxyapatite crystals than wild type animals [19]. It is probable that osteocytes elaborate OPN to inhibit the growth of hydroxyapatite in the wall of their lacuna and in the canaliculi to avoid burying due to secondary mineralization. OPN is also produced and localized in the kidney, and more particularly in epithelial cells of the convoluted distal tubule, a region with a high propensity for spontaneous precipitation of calcium salts. In this particular location, OPN is released in urine to prevent spontaneous precipitation of calcium salts [20].

It is well known that histochemical mechanisms of the silver stainings are complex. Argyrophilia probably occurs in two stages, namely, an initial attachment of silver to a reaction site on a protein, followed by nucleation of further silver on the original bound metal, giving a black appearance after silver reduction [21, 22]. The argyrophilic nature of nucleolar proteins is related to their N-terminal domain which are enriched with aspartic residues [23], similar findings have been described for SSP29, a leucin-rich repeat protein containing Asp residues [24]. OPN is a negatively-charged glyco and

phosphoprotein of approximately 300 amino acid residues containing a RGD cell binding sequence, a calcium and hydroxyapatite binding site, two heparin binding domains and a thrombin cleavage site. In addition to these structural elements, OPN is aspartic acid-rich and may be highly phosphorylated on serines and threonines, depending on the tissue, endowing the protein with a highly acidic character ($pI = 5$) [25, 26]. It is likely that the technical acid conditions of the AgNOR staining are responsible for its specificity. The acidity of the gelatin-silver bath appears to be a critical step which confers specificity to AgNOR binding [27]. Aspartic acid has also been found, by Raman spectroscopy, to bind silver due to its two carboxylic groups and the possibility to form the $HOOC-CH_2-CH(NH_3^+)-COO^-$ species at a low pH [28].

The AgNOR staining method appears to possess specificity for proteins containing aspartic acidic repeats in their N-terminal part. OPN was detectable in bone and kidney by AgNOR staining and immunohistochemistry at the same locations. In bone allografts extensively cleaned by industrial processes combining oxygen peroxide and sodium hydroxide, the AgNOR staining failed to identify this argyrophilic protein in the bone matrix, confirming the deleterious action of these compounds on bone proteins [29].

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LEGENDS OF FIGURES:

- Figure 1: AgNOR staining in lymphocytes from a benign (A) and lymphomatous (B) pleural effusion. Note the increase in NORs in the nuclei of malignant cells. Original magnification: x1000.
- Figure 2: AgNOR staining identifies osteocyte canaliculi, osteoplast boundaries and cement lines (arrow) in bone. Note the AgNORs in the nuclei of lining cells at the surface of the Haversian canal. Original magnification: x400.
- Figure 3: AgNOR granules in the cytoplasm of cells of the distal convoluted tubule of the kidney (arrows). Note the AgNORs in the nuclei of renal cells. Original magnification: x400.
- Figure 4: Immunolocalization of osteopontin in (A) the human bone matrix, (B) the kidney parenchyma. Note that silver-stained granules are only positive in the convoluted tubule. Original magnification: x400.

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