CHAPTER 17

Technical Aspects: How Do We Best Prepare Bone Samples for Proper Histological Analysis?

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I. INTRODUCTION

Histological analysis of bone is a critical step for the diagnosis of malignancies. It allows a direct identification of malignant cells inside marrow spaces in the case of bone metastases or hematological disorders. Bone biopsy is superior to marrow aspiration because the microarchitecture of the bone marrow is preserved, a parameter that is especially important in hematological disorders. Because marrow cells are in direct contact with bone cells (lining cells, osteoblasts, osteoclasts and their precursors) an abnormal bone remodeling rate has been described in a variety of malignant cell proliferations when developing and expanding inside marrow spaces. Bone cells elaborate and synthesize a variety of cytokines acting on hematological precursors (e.g. M-CSF) [1] and malignant cells release other cytokines active on bone remodeling [2–4]: it is likely that bone changes are almost always associated with bone marrow alterations and vice versa. Histomorphometric analysis is a powerful tool in the evaluation of bone remodeling in metabolic bone diseases and has also been successfully applied to hematological disorders and metastases from solid tumors [5,6]. Bone histomorphometry is a powerful method in the early diagnosis of B-cell malignancies and smoldering myeloma or lymphomas can be characterized in patients with a monoclonal gammopathy of undetermined significance (MGUS) several years before the tumor has a clinical expression. Bone histomorphometry is also useful in animal models of cancer bone lesions since it permits a precise evaluation of the bone remodeling changes induced by tumor cells [7–9]. However, bone histomorphometry must be done on undecalcified bone sections which allow a perfect identification of osteoid tissue (the unmineralized bone matrix recently synthesized by osteoblasts), a precise identification of osteoclasts (by using histoenzymatic detection) and histodynamic analyses (after a double tetracycline labeling in humans or using a variety of other fluorochromes in animals). There methods cannot be done on decalcified and paraffin embedded bone since decalcification abolishes the osteoid/bone matrix differential staining, removes the fluorochrome labels and hot paraffin embedding destroys enzyme activities. However, decalcification and paraffin remains useful for immunohistochemistry which is difficult and hazardous on plastic sections. The main disadvantage of polymer embedding was formerly the prolonged time for preparing bone specimens (several months were needed when polyester resins were used). With the development of histological techniques, it is now possible to have polymer embedding methods which are as fast as conventional paraffin methods. The following techniques have been developed and improved in our laboratory during the last two decades and used on more than 3000 human bone biopsies and a large number of animal studies performed in a variety of animal species (mouse, rat, chicken, dog, goat, sheep, pig).

II. BONE BIOPSY IN HUMANS (OR LARGE ANIMALS)

As recommended since the 1970s, bone trephines with a large inner diameter must be used: a 7mm trephine is
necessary to preserve bone microarchitecture and to analyze a representative amount of marrow spaces. We have proposed several modifications concerning ergonomics of the Meunier’s trephine making it easier to handle and providing better preserved bone cores [10]. The trephine developed in our laboratory is proposed by Commeca (Commeca, Beaucouzé-Angers, France) (website at http://www.commeca.com/anglais/cadresoma.html). Bone biopsy in human patients is painless when done with a double cortical anesthesia. The technique (including video) is described elsewhere (http://www.med2.univ-angers.fr/discipline/lab_histo/bone_biopsy.htm).

III. BONE FIXATION

Classically the collagenous bone matrix is said to be better preserved in a 70° ethanol fixative. However, it induces marked cell shrinkage incompatible with cytological examination. Formalin fixation provides very good cell preservation but induces poor staining of the collagen when using a trichrome method. The combination of both ethanol and formalin was proposed by Beebe and works perfectly well [11]. The formula known as BB’s fluid is:

- 95° ethanol – 900 ml
- 37–40% formaldehyde – 100 ml
- Deionized water – 150 ml

Bone biopsies are fixed for 24 hours at 4°C in a refrigerator. Then the fixative is discarded and replaced by acetone. BB’s allows the preservation of bone cell enzymes and retains the staining properties of collagen. Fixation in the cold (4–8°C in a refrigerator) improves fixation. After 24 hours, the fixative is discarded and replaced either by acetone or by the fast dehydrating fluid as above.

IV. MICROCOMPUTED TOMOGRAPHY (MICROCT)

MicroCT is a new microscopic technique developed over the last few decades [12,13]. It is a miniaturized version of computed tomographs commonly used by radiologists and the system now has a resolution in the order of 2 μm. MicroCT are based on a sealed microfocus X-ray source, a CDD camera and a step-by-step platform which receives the bone samples. Bone biopsies or animal bones can be analyzed when still in the fixative. They are transferred into an Eppendorf test tube filled with polyester fibers impregnated with the fixative (which are radiolucent and immobilize the samples). MicroCT scans are obtained within an hour, reconstruction of images, 3D model building and morphometry are done within 2 hours in human or animal samples [6,14]. Examples of microCT images appear on Figure 17.1A, B. They are useful as an early diagnostic method since sclerotic and osteolytic changes can be identified rapidly.

V. DEHYDRATION AND INFILTRATION

A rapid dehydration and defatting procedure is done by combining acetone and xylene at the same time [11]. Acetone is preferred to ethanol since it does not inactivate bone cell activities. We found that using a rotative device (running at a low speed) considerably shortens the dehydration time (Figure 17.2A). Bone biopsies are transferred in screw-capped test tubes that allow complete infiltration. Dehydration and defatting is accomplished during 3 hours in three consecutive baths (1 hour each). A final bath in pure xylene (1 hour) will ensure complete clearing.

VI. BONE EMBEDDING

Formerly, bones were embedded in polyester resins. Araldite and epoxies have also been proposed. However, the high viscosity of these monomers necessitates prolonged
infiltration times (more than 3 weeks, a time that is not compatible with routine diagnosis in cancer patients). Methylmethacrylate (MMA) based embedding fluids are now favored because the monomer has the same viscosity as water and diffuses rapidly inside the bony tissues. However, a number of mistakes and handling errors are common in laboratories when working with MMA (and its polymerized form pMMA) and the following simple rules can be recommended.

A. Purification of MMA

Purification of commercial MMA is always necessary when reliable results are needed. The monomer always contains a polymerization inhibitor (usually hydroquinone or 4-methoxyphenol) added by the manufacturers to avoid auto-polymerization. Several methods are available but the simplest consists of using several washes in 1M NaOH as follows [15]: one liter of crude MMA is mixed with 500 ml of 1M NaOH in water by using a separator funnel. The mixture is shaken vigorously for 2 min and then let to separate. Because MMA is hydrophobic, two phases separate: the lower phase contains water, NaOH and brownish oxidized phenolates; the upper is MMA with residual water droplets in suspension. The lower phase is withdrawn and replaced by 500 ml of fresh NaOH and shaken as above. This lower phase is then discarded and replaced by deionized water, shaken and the aqueous phase is discarded once more. At that time, the MMA phase is cloudy (due to remaining water droplets in suspension); it is transferred into a screw capped bottle and placed in a freezer at –20 °C. By the following day, ice crystals will have formed and are separated from MMA by vacuum filtration using a Büchner funnel with a Whatman no. 3 filtration paper. MMA free of inhibitor is stored at –20 °C in screw capped bottles until use.

B. Preparation of the Accelerated MMA Medium for Infiltration and Embedding

The embedding medium is prepared by mixing 12 g of benzoyl peroxide BPO with purified MMA 1000 ml using a magnetic stirrer. Since BPO is explosive when dry, it is always stabilized with large amounts of water. These traces of water are eliminated by refreezing at –20 °C and elimination of ice crystals by a new vacuum filtration [15]. A plasticizer (usually dibutylphtalate) is then added (100 ml for 900 ml of activated MMA) and the infiltration medium is then stored at –20 °C to avoid BPO decomposition. An initiating mixture (to induce redox polymerization) is prepared by mixing 2 ml of the tertiary amine (N-N-dimethylaniline) with 18 ml of 2-propanol. This initiating solution is kept at +4 °C in a refrigerator.

C. Fast Infiltration and Embedding Method

We found that a gentle constant agitation also considerably shortened the infiltration time. The test tubes containing the bone samples (dehydrated and cleared as above) are then filled with 10 ml of the accelerated MMA mixture and placed on the rotative machine for 1 hour. The mixture is then changed and left rotating for an additional hour at room temperature. A third and final bath is then prepared by using an accelerated and initiated MMA bath:

- Accelerated MMA mixture – 25 ml
- Initiating solution – 400 μl

It is recommended that Gilson Pipetman® with disposable polyethylene tips is used for handling MMA and initiating mixtures.
The bone samples immersed in the accelerated and initiated MMA are then stored in a freezer at −20°C overnight. This ensures a complete and uniform diffusion of all components inside bone.

D. Embedding

Former authors used embedding in glass vials, a dangerous method since block retrieval necessitates breaking the vial with a hammer. We have found that polyethylene molds (Peel-a-Way embedding system, Polyscience Inc, Warrington PA) constitute a well adapted disposable system. A strip of polyester sponge or a piece of polyester wadding (used in aquarium or kitchen hood filters) is placed at the bottom of the mold to avoid the interface-linked shrinkage during polymerization [16,17]. Air contact is prevented by placing a thin film of Parafilm® (Alcan, Packaging, Neenah, WI) since atmospheric oxygen is a strong polymerization inhibitor [18]. The Peel-a-Way® molds are placed in a water bath at 4°C for 24 hours and polymerization slowly proceeds in the cold. Decomposition of BPO by NN dimethylanilin is an exponential function of the temperature (Arrhenius’ law): at −20°C, polymerization is inhibited (but infiltration is favored); when the temperature is raised at +4°C, the redox polymerization starts [19]. The water bath is used to limit the exothermic peak associated with MMA polymerization. This preserves enzyme activities (e.g., tartrate resistant acid phosphatase (TRAcP). Embedding and polymerization under vacuum is senseless since this increases MMA evaporation, and increases the number of holes inside objects (note that the polymerization defects often referred to by technicians are not gas bubbles but holes created by the shrinkage of the embedding mass during polymerization) [17].

E. Sectioning

Sections are cut at 7 μm using a heavy duty microtome (e.g. Leica, Polycut-S) equipped with 50–60° tungsten carbide knives for histomorphometry. Sections 14 μm in thickness are used for tetracycline (or calcein) histodynamic studies. Flat sections can be obtained with a strip of paper and a forceps (Figure 17.2B and C).

VII. STAINING METHODS

A. Osteoid Tissue and Calcified Bone

Masson, Mallory or Goldner’s trichrome are favored for the identification of osteoid/mineralized matrix. However, classical procedures stain osteoid and marrow cells with the same red intensity, making osteoid difficult to analyze accurately. We have designed a special trichromic method which considerably improves the differentiation of osteoid from the bone narrow [20]. Sections are stained by free floating in glass vials containing the staining solutions. A ‘rock-and-roller’ device allows homogeneous staining and avoids sections staying in contact with the sides of the vial (Figure 17.2D).

- Stain for 30 min in a saturated picric acid solution (1.2 g in 100 ml of water)
- Wash 30 sec in deionized water
- Stain for 15 min in Meyer’s hematoxylin (Merck)
- Rinse in deionized water, blush in a saturated lithium carbonate aqueous solution (5 g in 1000 ml of water), rinse in deionized water
- Stain for 15 min in the following solution:
  - Acid fushine – 0.1 g
  - Xylidine ponceau – 0.4 g
  - Deionized water – 100 ml
  - Glacial acetic acid – 1 ml
- Wash in 1% acetic acid for 30 sec
- Differentiate for 1 min in:
  - Orange G – 1 g
  - Deionized water – 100 ml
  - Phosphomolybdic acid – 1.5 g
- Do not wash, counterstain in the following solution:
  - Fast green FCF – 0.1 ml
  - Deionized water – 200 ml
  - Glacial acetic acid – 1 ml
- Rinse in 1% acetic acid
- Dehydrate in 2-propanol (2-propanol is a dehydrating solvent that has no effect on pMMA sections; on the contrary mixture of water-ethanol can softened considerably the sections)
- Transfer into three successive baths of methylcyclohexane or Histolemon® (these compounds are non-solvent for pMMA) (Figure 17.2E)
- Mount in a synthetic medium (e.g. Neo Entellan®, Merck)
- Flatten sections by compression between two wood pieces for 12 hours (Figure 17.2F).

The use of picric acid considerably enhances the red staining of osteoid tissue and reduces marrow cell staining; nuclei are faintly stained (Figure 17.3; see Plate 6). Other old methods for the identification of osteoid on undecalcified sections are not described: Von Kossa is known to be less precise than trichromes and is not specific for calcium [21]. Solochrome cyanin has a poor lightfastness.

B. Argentophilic Proteins (AgNOR Method)

Argentophilic proteins are found in the nucleoli of all cells (nucleolin, RNA polymerase I, protein B23) where they constitute the Nucleolar Organizing Regions (NOR). We found that osteopontin, a non-collagenic protein of the bone matrix rich in Asp residues, was also stainable by the AgNOR method [23,24]. The following technique can be recommended. Undecalcified sections need to be decalcified for 1 hour in 10% aqueous formic acid before staining to avoid substitution of calcium (of the bone matrix) by silver atoms. Sections are incubated for 55 min at room
temperature in the dark, in a staining solution prepared by combining silver nitrate (2 volumes of 50% aqueous solution) with formic acid (1 volume of a 1% solution containing 2% gelatin). After staining, sections are thoroughly washed in deionized water and transferred for 10 min to a 5% aqueous sodium thiosulfate solution prepared ex tempore. Sections are then rinsed with deionized water dehydrated and mounted as above.

AgNORs appear as black dots in the nucleoli of all cells. The reaction is clearly evidenced in marrow, cancer and bone cells and the number of NORs is known to be increased in cancer cells [25]. On bone sections, dense stripes of argentophilic material are found around the osteocytes and canaliculi and at the periphery of their lacunae (Plate 6E). The delicate inter-osteocyte relationships are clearly evidenced. Linear deposits are also observed in the matrix and correspond to the cement and resting lines.

C. Histoenzymatic Identification of Osteoclast by TRAcP Staining

TRAcP is a thermo-sensitive enzyme which is also destroyed by prolonged fixation in formalin or alcohol. The above-mentioned procedures for fixation and embedding ensure a perfect preservation of TRAcP activity in osteoclasts [15,26]. The following procedure is well adapted to histomorphometry because the naphtol phosphate used tends to provide a homogeneous staining of the osteoclast cytoplasm [27]. Substituted naphtols (AS-BI, AS-TR) can be used but they provide a more precise localization of the enzyme inside intracytoplasmic lysosomes, making identification of osteoclast more difficult at low magnification. The staining method is a follows:

- Prepare an acetate buffer by mixing:
  - Sodium acetate, 3H2O – 19 g
  - Glacial acetic acid – 4.5 ml
  - Deionized water – 1000 ml
  - Sodium tartrate – 150 mg
- Prepare solution A which is the enzyme substrate:
  - a-naphthyl phosphate, Na salt – 100 mg
  - Acetate buffer, pH 5 – 100 ml
- Prepare solution B which contains the dye:
  - Fast Violet B salt – 250 mg
  - Acetate buffer, pH 5 – 50 ml
- Mix both solutions and filter immediately
- Incubate freshly prepared bone sections by free floating at 37°C for 60 to 90 min
- Rinse in deionized water
- Inactivate the remaining enzymatic molecule by incubation for 60 min in:
  - Sodium fluoride – 4.2 g
  - Deionized water – 1000 ml
- Rinse in deionized water
- Counterstain for 20 min in:
  - Anilin blue WS – 66 mg
  - Phosphotungstic acid – 2 g
  - Deionized water – 200 ml
- Rinse in deionized water and mount in Apathy’s syrup.

Because the condensation product between the naphtol and the diazonium is soluble in water, sections must be mounted in an aqueous medium. Synthetic media often contain alcoholic groups or other functions that destroy the colored precipitate. Currently, the best known medium is Apathy’s syrup prepared, in large quantities using centrifugation to eliminate minute residual air bubbles [28], as follows. Briefly, powder of arabic gum (500 g) and saccharose (500 g)
are placed in a bottle containing deionized water (500 ml) and a large thymol crystal. The bottle is then closed with a screwed plug and transferred in a drying oven at 65°C for 48 hours. It is regularly agitated every 4–6 hour period. A viscous brown syrup is obtained and distributed into 25 ml centrifuge tubes. The tubes (containing hot syrup) are then centrifuged at 2000 rpm for 30 min. The very fine residual layer of bubbles which persists at the end of the centrifugation is then eliminated with a spatula. The syrup is then distributed into 20 ml syringes. This syrup can be preserved for more than 1 year in the refrigerator.

D. Mast Cell Identification in Mastocytosis

Mast cells contain granules rich in sulfated GAG (such as heparane sulfate), histamine, cytokines and proteases (tryptase). The sulfated GAGs confer metachromasia to the mast cell granules. In systemic mastocytosis, these cells are increased in number; they can form nodules in the bone marrow and can adhere to osteoblast or lining cells at the surface of bone trabeculae. Because mast cells can contain a few granules in some types of mastocytosis, a sharp contrast is obtained using the following method:

- Stain section by free floating for 15 min in:
  - Toluidine blue – 0.5 g
  - Deionized water – 100 ml
  - Glacial acetic acid – 1 ml
- Rinse in 1% acetic acid until no more blue stain is extracted from the sections
- Dehydrate in 2-propanol and mount as above.

Granules of mast cells appear deep violet and the tint of the cell depends on the number of cytoplasmic granules.

E. Living Osteocytes in the Bone Matrix

Osteocytes are mechanosensitive cells responsible for maintaining bone tropicity. These cells can disappear inside their lacunae and it is thought that bone packets devoid of osteocytes can be removed by osteoclasts. Living osteocytes can be detected inside the bone matrix by using a highly fluorescent nuclear stain associated with a fluorescent counterstain for the bone matrix. The following method works remarkably well.

Stain nuclei during 30 min with Hoechst 33342 prepared as follows:

- Hoechst 33342 – 2 mg
- Deionized water – 1000 ml
- Rinse in deionized water

Counterstain during 10 min in:

- Nuclear yellow fast R – 1 g
- Formalin – 100 ml
- Phosphotungstic acid – 1 g
- Rinse in deionized water and mount in Apathy’s syrup.

Hoechst 33342 has been used widely as a fluorescent dye for staining the nuclei of living cells. It preferentially binds to AT regions of DNA and shows no cytoplasmic staining. Observations and measurements are made under UV fluorescence microscopy with a WU near-ultraviolet fluorescence cube. Osteocytes appear as intensely stained green spots (and only intact nuclei are stained) on a restained background corresponding to the calcified bone matrix. Nuclei of bone marrow cells, endothelium and lining cells are also stained.

These staining methods have been developed over the last few decades. The success of many of them depends on the use and quality of the pMMA embedding in the cold. The use of pMMA as an embedding medium allows identification of a large number of details or structures that cannot be identified on decalcified and paraffin embedded bones. However, immunohistochemical studies are difficult and only a few reports have been presented. Undecalcified embedding methods have provided a number of clinically and scientifically reliable reports and have considerably changed the evaluation of benign and malignant bone diseases.

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References