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Techniques to study cellular response in critical size bone defect healing on rat calvaria treated with hydroxyapatite implants

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Abstract. The aim of this paper was to evaluate the usefulness of coupling digital image analysis with immunohistochemistry and histomorphometry data to the study of tissue response to hydroxyapatite in a model of critical size bone defect in calvaria of rats. A transosseous defect measuring 8 mm in diameter was performed with a surgical trephine in the parietal bone of 40 rats and divided into two experimental groups according to the treatment: group I (blood clot, control), group II (HA) and killed 1, 3, 6 and 9 months after implantation (n=5/group/period). The skullcaps with overlaying skin were collected and processed for paraffin embedding. The specimens were cut in the laterolateral direction into 5-µm thick semi-serial sections and stained with hematoxylin-eosin for identification and counting of polymorphonuclears cells, mastocytes, and multinucleated giant cells, MNG, or immunolabeled with anti- lysozyme, -factor VIII and –PCNA. Digital images were obtained and analyzed with the ImagePro-Plus® software for cell couting (polymorphonuclears cells, mastocytes, macrophages and MNG) and microvessel density. Image segmentation of anti-PCNA immunostaining was used for cell proliferation analysis. The digital images obtained allowed clear identification of cells of interest by through morphological aspects or immunostaining. Data recording and analysis was facilitated by the use of specific software for image processing and graphical and statistical analysis. It can be concluded that the techniques applied were usefull to identify and count cells, structures and process of interest making easier the effectiveness of hydroxyapatite in the critical size defect in rat calvaria model.

Introduction

Host response to implanted biomaterials may include trauma, blood-biomaterial interaction, provisional matrix production, acute inflammation, chronic inflammation, establishment of a granulation tissue, foreign body reaction and fibrous capsule [1].

Blood coagulation starts the inflammatory stage of wound healing and the diffusion of chemoattractants (growth factors from platelets) attracts polymorphonuclears neutrophils (PMN), followed by monocytes, from blood. In parallel to cell migration and proliferation, cells (fibroblast) synthesize extracellular matrix (ECM) proteins depositing it from the margin of the wound inward. The connection of ECM and cell metabolism through integrins seems to regulate cell function. PMN and mononuclears cells characterize the acute and chronic phases of inflammation.

Histamine and heparin release from mastocyte controls key events in the inflammatory response to implanted biomaterials, as well the FBR intensity by interleukins mastocyte-derived [2]. Monocytes migration and accumulation of macrophages, lymphocytes and plasma cells establish the chronic phase, being the macrophages and their function fundamentals for the transition from inflammatory to the proliferative stage of wound healing. The normal wound healing response to implanted biomaterials show a foreign body reaction, formation of granulation tissue and angiogenesis from proliferating endothelial cells [3, 4]. Particularly for bone grafts, the angiogenesis plays a pivotal role in the osteogenesis and osseointegration delivering pericytes able to differentiate in osteoblasts [5]. The microscopic evaluation of host-biomaterial interaction allows the identification of cells and ECM changes induced by biomaterials. Histological sections stained by hematoxylin (H) revels the cell nucleus (blue or violet) and other acidic structures (RNA rich cytoplasm regions or hyaline cartilage). On the other hand, eosin (E) makes the cytoplasm and collagen pink [6]. However, HE per se was not enough to morphological identification of all type of cells or process. Immunohistochemistry improves the identification of cells and cellular processes by the reactions of antibodies and antigens associated to chromogens [7, 8]. The antibodies could be polyclonal or monoclonal, but the monoclonal possess greater specificity. In addition, they can also be classified as primary or secondary agents, being the first raised against the epitope of interest. The second recognizes immunoglobulins and are conjugated to biotin, enzymes (alkaline phosphatase or horseradish peroxidase) or fluorescent tags. Choosing the correct antibody and reaction conditions are essential for unbiased analysis.

Comparative analysis of biomaterials is routine in the biomaterials field. To minimize the bias of subjective analysis, histomorphometry are preferred, and determines lengths, perimeters, areas, and benefits of image analysis software's facilities [9]. It is necessary to study the tissue response of bone substitutes. Broadening the understanding of the complex cell/material interactions will contribute to the development of novel biomaterials since tissue-engineered constructs that direct biological responses [10, 11].

The aim of this paper was to evaluate the usefulness of coupling digital image analysis with immunohistochemistry and histomorphometry data to the study of tissue response to hydroxyapatite in a model of critical size bone defect in calvaria of rats.

Materials and Methods

Forty Wistar rats (5 months old) which were submitted to 8 mm diameter critical size defect in the skull were divided into two experimental groups according to the treatment: group I (blood clot, control), group II (HA) and killed 1, 3, 6 and 9 months after implantation (n=5/group/period). All surgical procedures followed the guidelines of Brazilian College of Animal Experimentation (COBEA) and were approved by local IRB. The skullcaps with overlaying skin were collected and processed for paraffin embedding. The specimens were cut in the laterolateral direction into 5-µm thick semi-serial sections and stained with HE for identification and counting of polymorphonuclears cells, mastocytes, and MNG. Photomicrographies were taken by a digital camera digital coupled with an optical microscope with 100x lens. Polimorphonuclears, mast cells and MNG were identified and quantified by images of about 40 photomicrographies taken for each histological section resulting in 1600 digital images without superposition.

The sections were arranged in slides coated with polylysine and submitted to the indirect immunoperoxidase method [8]. Immunohistochemistry was used to identify and quantify macrophages (anti-lysozyme, 1:600, Dako, Dinamarca, A 0099), micro vessel density (anti-factor VIII, 1:400, Dako, Dinamarca, A 0082) and cell proliferation intensity (anti-PCNA, 1:400, MBL, EUA, JM 3350R-100). Photomicrographies of histological sections treated with anti-lysozyme and anti-factor VIII were taken by digital camera connected to a optical microscope with 40x lens. Ten photomicrographies taken for each histological section resulted in 800 digital images without superposition. Ten photomicrographies taken for each histological sections immunostained with anti-PCNA were obtained in the same system but with 20x lens resulting in 400 digital images without superposition. The digital images for histomorphometry were analyzed in the Image

ProPlus® (Media Cybernetics, L. P., Silver Spring, MD) program. The cell proliferation intensity was determined by image segmentation. Sections immunostained with anti-factor VIII analyzed through a 50 x 50 µm reference grade aided the microvessel density determination since only vessels with a internal caliber lower than 50 µm were counted.

Results and Discussions

PMN (Fig. 1A), mastocytes (Fig. 1B), macrophages (Fig. 1C) and MNG (Fig. 1D) were identified by its morphology and quantified. Automatization of morphological analysis is still a challenge since the structural, dimensional and staining complexity of biological sections makes difficult the correct identification of the target structure. PMN are phagocytic acute phase inflammatory cells responsible for removal of substances and microorganism from injured tissue. Mastocytes are present at all phases of wound healing and affects collagen synthesis and vessels permeability [12]. Acute to chronic inflammation transition is marked by the macrophage recruitment able to degrade bacteria cell wall by the action of lysozyme [10] and, once adhered to biomaterial surface, they could merge to form MNG cells [13]. However, the presence of multinucleated giant cells and a foreign body-type inflammatory reaction are known to markedly inhibit new bone formation [14]. Among several cytokines, IL-4 plays a central role in the generation of MNG that is dependent of STAT6 expression [15].

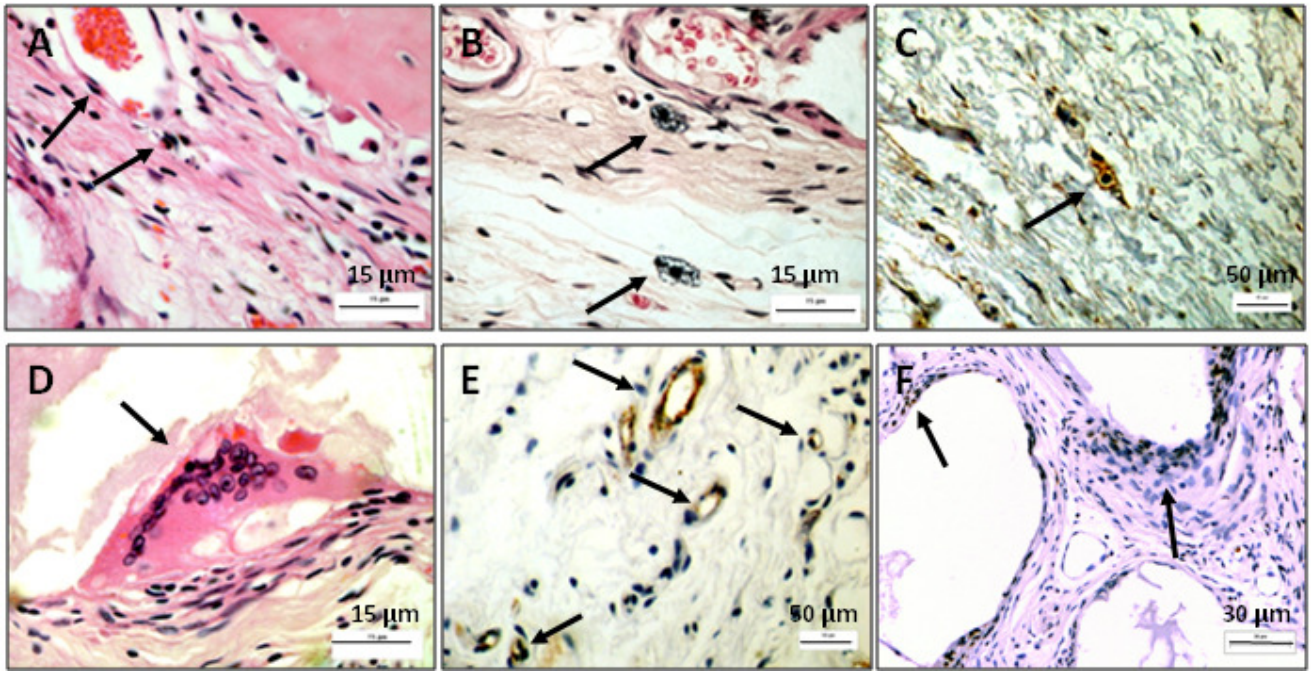

Figure 1: Photomicrographies of histological sections . A) Polimorphonuclears (arrow); B) Mast cells (arrow); C) Macrophages (arrow); D) MNG (arrow); E) Microvessel density (arrow); F) Proliferating cells (arrow).

There are many scientific hypotheses regarding the nature of inflammatory MNG attracted to subcutaneous grafts of mineralized particles [16], in opposition to the idea that these cells were osteoclasts with ruffled borders [17]. Two types of multinucleated cells may be observed surrounding mineralized bone grafts. One type has a typical morphology of an MNG and evidence of bone degradation, and another, in smaller number, has cytological characteristics of an osteoclast but without the genuine ruffled borders [18]. For these authors resorption may occur by means of either an MNG or an osteoclast. In a previous study, we verified the presence of such IMGCs in contact with the implanted material, however, the presence of brush-like borders was not observed [19], corroborating previous work that showed MNG recruitment in response to mineralized homograft but without osteoclast characteristics [16].

The analyzed structures were not often clearly distinguished from the background and hadn't a color intensity scale sufficiently different from other non relevant structures. However, the quantification of cell proliferation intensity could be done by image segmentation since only proliferating cells are positive to PCNA [20] (Fig. 1F). In addition, all immunopositive cells were analyzed irrespective to their dimension and structure. To estimate the angiogenesis level, blood vessels with internal caliber shorter than 50 μm were counted (Fig. 1E), as has been done in the evaluation of angiogenesis in tumors [21]. In the present work, the identification and quantification of targeted microvessel was very simple, and could be a useful tool to study the angiogenesis induced by biomaterials.

Conclusions

We concluded that immunohistochemistry and histomophometrical analysis coupled with digital image analysis improved the evaluation of tissue response to hydroxyapatite in critical size bone defect healing.

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