COMPARATIVE IMMUNOHISTOCHEMICAL STUDY ON OSTEOCALCIN EXPRESSION IN FIBROUS DYSPLASIA AND OSSIFYING FIBROMA

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ABSTRACT

Objective: Fibrous dysplasia (FD) and ossifying fibroma (OF) show similar histological and radiological features, making distinction between them a diagnostic dilemma requiring clarification. Study Design: Forty (20 FD, 20 OF) paraffin-embedded specimens were immunohistochemically stained using the primary antibody Osteocalcin (OC). The immunostained area fraction was evaluated in the bone trabeculae alone and in both the bone trabeculae and the connective tissue stroma together. Results: All cases of FD and OF demonstrated OC immunostaining in both the bone trabeculae and the stromal cells. In FD, osteocalcin was more expressed in stromal cells, while in OF, immunoreactivity was more marked in bone trabeculae. The area fraction expressing OC in both the bone trabeculae and fibrous stroma was significantly higher in FD than OF (p= 0.026). Conclusion: The stromal spindle-shaped cells in FD are of the osteoblastic lineage. Osteocalcin expression can be a significant support in differential diagnosis of FD and OF.

Key words: Osteocalcin, Fibrous dysplasia, Ossifying fibroma

1. INTRODUCTION

Benign fibro-osseous lesions of the jaws constitute a varied group of lesions with a common histological characteristic: the substitution of normal bone by tissue composed of collagen fibers and fibroblasts, with variable amounts of a mineralized substance that may be bone, cementum or both. Among these lesions are fibrous dysplasia (FD) and ossifying fibroma (OF), both showing similar histological and radiological features, thus making distinction between the two a diagnostic dilemma.

FD represents a bone developmental disorder, manifesting a defect in osteoblastic differentiation and maturation. This dysplastic process is characterized by self-limited growth and apparent responsiveness to the hormonal changes of puberty.

Cemento-ossifying fibroma is a benign fibro-osseous lesion of the jaws that consists of cellular fibroblastic tissue containing varying amounts of osteoid tissue, bone, cementum, and cementum like calcified tissue. This lesion seems to arise from the periodontal membrane, which contains pluripotential cells capable of forming cementum, bone, and fibrous tissue. Ossifying fibroma is relatively slowly growing that may be present for a number of years before a diagnosis is made.

Anubha et al. stated that the dilemma in diagnosis of FD and OF rests in the bony trabeculae as well as in the fibrous stroma, where cases of FD, showing lamellated bony trabeculae and osteoblastic rimming have been reported and may confound diagnosis because of resemblance with OF. In their study, an attempt has been made to demonstrate the fibrous element of these two lesions using histochemical stains and the study revealed that the oxytalan fibers were more numerous in OF.

According to Toshihisa, type I collagen, osteonectin (ON), osteopontin (OP), and osteocalcin (OC) are bone matrix proteins. While also being phenotypic markers, they have characteristics that are often associated with osteoblastic differentiation. ON and OP appear in the early stages of osteogenic maturation, whereas OC appears in the late stages.

OC is a vitamin K-and vitamin D-dependent protein that has high affinity for calcium and exhibits a compact calcium-dependent α-helical conformation. Osteoblasts synthesize OC which after production is partly
incorporated into the bone matrix and partly delivered to the circulatory system. Circulating OC is associated with changes in the rate of bone turnover and regarded as a specific marker for bone formation.\(^{16}\)

The physiological role of OC is not precisely understood, but OC does serve as an inhibitor of crystal growth in vitro and has been postulated to play a role in osteoclast recruitment and bone resorption. Osteocalcin deposition in bone matrix is most likely a hallmark of mature, metabolically inactive bone, whereas OC itself may be a cell marker for osteoblastic differentiation (Sakamoto et al, 1999).\(^{11}\) Immunohistochemical reactivity for OC was observed in the cytoplasm and plasma membranes in the stromal cells, calcified structures, and bone surrounding osteoblasts in neoplastic and bone-related lesions (Elias et al, 2010).

Since fibrous dysplasia and ossifying fibroma show distinct patterns of disease progression, it is important to distinguish between the two. Therefore, this study was conducted to analyze the immunohistochemical expression of osteocalcin in FD and OF, in order to assess its potential role in differentiation between these two disease entities.

2. MATERIAL AND METHODS

Specimens collection

Thirty formalin-fixed, paraffin-embedded specimens were included in the present study. These included 20 cases diagnosed as FD and 20 cases diagnosed as OF. All cases were collected from the archives of the National Cancer Institute and from the Oral Pathology Department, Faculty of Oral and Dental Medicine, Cairo University. Hematoxylin and eosin stained sections were used for confirmation of the diagnosis.

Immunostaining Procedure:

Four-μm-thick sections were deparaffinized with xylene, and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by immersion of slides in methanol with 0.03% hydrogen peroxide for 30 min. Sections were then microwaved in citrate buffer, pH 6.1, at 95°C for 10 min for antigen retrieval. Non-specific binding was blocked with 3% normal rabbit serum in phosphate-buffered saline, (DAKO, Glostrup, Denmark). The lysophilized primary antibody: Osteocalcin Rabbit anti-Human Polyclonal antibody (MyBioSource, LLC, San Diego, USA) was diluted in phosphate buffer saline (PBS), (10 mg antibody/ml PBS). Sections were incubated with the primary antibody overnight, then were stained according to the avidin-biotin complex method using the ready-to-use Ultravision Detection System Anti-Polyvalent (Lab Vision Corp., USA) and visualized using 3,3-diaminobenzidine (DAB). The specimens were subsequently counterstained with Mayer’s hematoxylin.

Negative control sections were processed identical to experimental sections except that the primary antibody was omitted and replaced with phosphate buffer saline.

Immunohistochemical Evaluation:

In each slide, 5 microscopic fields showing the highest immunoreactivity were selected and photomicrographed. Images were acquired using a digital video camera (Olympus, C5060, Japan), which was mounted on a light microscope (Olympus, BX60, Japan). The immunohistochemical reaction was evaluated by the image analysis software (Image J, 1.37v, NIH, USA). Computerized calculation of the total surface area of the immunoreaction was expressed as a fraction (percentage) of the total surface area of the microscopic field (immunostained area fraction, IAF). The immunostained area fraction was calculated twice, one time including both the bone trabeculae and the connective tissue stroma, whereas, the second calculation was made after clearing the fibrous tissue (to evaluate immunoreactivity confined to the bone trabeculae). The used method constitutes a time efficient and reproducible method for quantification of the immunoreaction.

Statistical assessment

Using the Statistical Package for Social Science (SPSS 15.0) Software, Student t test was performed for comparison of means. Comparison was made between immuno-positivity in bone trabeculæ alone in FD and OF. Moreover, immunoexpression in both bone trabeculæ and stroma in FD and OF were compared. The results were considered significant when p value was ≤ 0.05.

3. RESULTS

(I) Immunohistochemical findings:

All cases of FD (100%) demonstrated homogenous brown OC immunostaining positivity in both the bone trabeculæ and the stromal cells. Immuno-positivity was more marked in the stromal cells than in the bone trabeculæ (Fig. 1). Cells close to the bone trabeculæ showed higher immuno-positivity than those away from the bone trabeculæ (Fig 2). OC expression was observed within the bone matrix, in osteocytes entrapped in bone trabeculæ and in cells bordering the thin irregular Chinese letter-like trabeculæ (Fig 3).

All cases of OF (100%) showed OC immuno-positivity in both bone trabeculæ and stromal cells; however the reaction was higher in the bone trabeculæ than the stromal cells (Fig 4). OC expression was observed within the bone matrix (Fig 5), in osteocytes entrapped in lacunæ and in the cytoplasm of the neoplastic fibroblasts (Fig 6).
Fig. 1. Photomicrograph of Fibrous dysplasia revealing more marked osteocalcin immunoexpression (OC) in the fibrous stroma than in the bone trabeculae (Osteocalcin x 200).

Fig. 2. Photomicrograph of Fibrous dysplasia revealing osteocalcin immunoexpression in the spindle-shaped cells of the fibrous stroma closer to the bone trabeculae (arrows). (Osteocalcin x 200).

Fig. 3. Photomicrograph of Fibrous dysplasia revealing osteocalcin immunexpression in osteocytes entrapped in bone trabeculae (red arrow), in cells bordering the trabeculae (blue arrows) and in spindle-shaped cells of the fibrous stroma (green arrows), (Osteocalcin x 400).

Fig. 4. Photomicrograph of ossifying fibroma illustrating more marked osteocalcin immunoreaction in the bone trabeculae than the stromal cells (Osteocalcin x 200).

Fig. 5. Photomicrograph of ossifying fibroma revealing osteocalcin expression within the bone trabeculae (arrows). (Osteocalcin x 200).

Fig. 6. Photomicrograph of ossifying fibroma revealing osteocalcin expression in osteocytes entrapped in lacunae within the bone trabeculae (red arrow) and in the cytoplasm of the neoplastic fibroblasts (green arrow), (Osteocalcin x 200).
**II) Image Morphometry Analysis:**
Comparing means of the area fraction of bone trabeculae showing OC immuno-positivity in both lesions revealed a higher mean in OF than FD, however, Student t test revealed that this difference was not statistically significant. On the other hand, the area fraction of both bone trabeculae and fibrous tissue stroma showing OC immuno-positivity was higher in FD than OF and the difference was found to be statistically significant (p= 0.025). (Table 1).

<table>
<thead>
<tr>
<th>In bone trabeculae</th>
<th>Ossifying fibroma</th>
<th>P value (Student’s t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Facial fibrous dysplasia</td>
<td>4.45±2.58</td>
<td>7.74±4.53</td>
</tr>
<tr>
<td>In bone trabeculae and fibrous tissue stroma</td>
<td>18.05±11.62</td>
<td>7.75±7.21</td>
</tr>
</tbody>
</table>

4. DISCUSSION

Regardless of subtype, all BFOLs demonstrate replacement of normal bone by fibrous connective tissue with an admixture of mineralized product, including osteoid, mature bone, and/or cementum-like calcifications. Thus, a histologic diagnosis of a BFOL, in many cases, is relatively complicated. Although OF and FD are typically benign, they affect the balance between bone resorption and apposition. So, they should be considered as belonging to the category of lesions which involve the process of bone remodeling. Since the exact mechanisms of bone resorption and apposition in these lesions are unknown, this study was conducted to immunohistochemically detect and compare the intracellular and extracellular localization of OC in FD and OF, in order to evaluate its potential role in differentiation between these two disease entities.

In the present study, a higher expression of OC in FD compared to OF was verified immunohistochemically in both the stromal spindle-shaped mesenchymal cells and the bone trabeculae in both lesions and the difference was statistically significant. The expression of OC in bone trabeculae of later stages of FD and in OF is a sign of bone maturation. Similarly, Sakamoto et al. observed that although OC was deposited to a large extent in metabolically inactive bone, whereas the matrix in newly apposed bone didn’t show any OC immunostaining. They suggested that OC deposition may be a relatively late event during the process of new bone formation. Moreover, the higher OC expression in bone trabeculae of OF compared to FD implies that bone trabeculae are more mature in the former lesion.

On the other hand, OC was expressed in the cytoplasm of stromal cells of FD. This finding accords with Elias et al., and emphasizes that these cells are osteoprogenitor cells. The osteoblastic nature of the stromal spindle-shaped mesenchymal cells in FD was demonstrated by electron microscopic analysis which revealed a lining of abnormal osteoblasts with a fibroblast-like appearance around the immature woven bone. Moreover, biochemical analysis showed alkaline phosphatase activity in cells populating the stromal area of FD, suggesting that these immature cells are actively depositing a woven bone matrix.

As explained above, the immuno-histochemical expression of OC in stromal cells of FD denotes that these cells are of the osteoblastic lineage, whereas the stromal cells of OF are fibroblasts involved in the formation of this benign neoplasm. Riminucci et al. reported that the spindle-shaped mesenchymal cells of FD share some phenotypic features with osteoprogenitor cells of normal osteogenic tissues, based on immunohistochemistry and in situ hybridization studies. On the other hand, Bartolini et al. suggested that OF develops from the multi-potential mesenchymal cells of periodontal origin which are able to form both bone and cementum. This suggestion also supported by Mintz and Velez. Further explanation was provided by Anubha et al. who stated that the fibrous connective tissue of the periodontal membrane is composed chiefly of collagen fibers, oxytalan fibers, mucopolysaccharides and cells which have the capacity to synthesize bone, cementum and fibrous tissue. Under pathologic conditions, suchastic cells are capable of producing tumors composed of cementum, lamellar bone and fibrous tissue.

A further possibility to be considered is that the stromal cells in OF are more immature, and therefore unable to express OC as suggested by Sakamoto et al., whereas the progenitor cells of FD are at a more mature stage of osteoblastic differentiation.

In addition, in the present study the immuno-positivity was more evident in the bone trabeculae than stromal cells in OF, while in cases of FD the immuno-positivity was evident in both stromal cells and bone trabeculae. The presence of moderate levels of OC in the bone trabeculae of FD may contribute to the characteristic disconnected appearance of bone trabeculae in that entity because OC is a negative regulator of bone formation. In contradiction, Elias et al. stated that bone resorption is favored in OF, in contrast to FD.

Moreover, in the current study, the immuno-positivity of bone trabeculae in cases of OF was seen in the bone matrix rather than the entrapped cells, while in cases of FD the immuno-positivity in bone trabeculae was evident in the entrapped cells as well as the bone matrix. According to Sakamoto et al., OC deposition in bone matrix is most likely a hallmark of mature, metabolically inactive bone, whereas OC itself may be a cell marker for osteoblastic differentiation. This hypothesis can be emphasized by the opinion of Ono et al., that maturation of osteoblasts can be determined to some extent by their expression of OC, since bone matrix formation and calcification depends on it. Toyosawa et al. as well concluded that the abundance of OC in FD and its deficiency in OF suggests that the calcified material in FD is more similar to normal bone than that in OF and this marked difference may indicate differences in bone formation and osteoblast differentiation between the two lesions.
5. CONCLUSION

When FD and OF were compared with each other, the stromal spindle shaped mesenchymal cells expressed more OC immuno-positivity in FD than OF. These findings suggest that the stromal spindle-shaped cells in FD are of the osteoblastic lineage and are mature stage of osteoblastic differentiation than those in OF. These data can be considered to be a significant support in differential diagnosis and furthermore suggest that FD and OF are distinct disease entities.

REFERENCES