

Expression of Insulin-Like Growth Factor-I (IGF-I) in Aneurysmal Bone Cyst

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The effects of insulin-like growth factor-I on bone tissue and its role in bone development have been extensively investigated, but there is little information on its role in the pathogenesis of aneurysmal bone cyst. Therefore, using the techniques of immunohistochemistry and *in situ* hybridization, the authors studied the expression of insulin-like growth factor-I in 19 specimens of aneurysmal bone cyst. Insulin-like growth factor-I or specific mRNA sequences encoding for insulin-like growth factor-I were detectable in all specimens tested and were mainly localized in multinucleate giant cells. In contrast, only insignificant levels of insulin-like growth factor-I expression were detectable in normal human bone tissue. Taken together with the previously reported role of insulin-like growth factor-I in the pathogenesis of giant cell tumor, the findings of this study suggest that insulin-like growth factor-I may play a role in the pathogenesis of aneurysmal bone cyst.

KEY WORDS: Aneurysmal bone cyst, Giant cell tumor, Immunohistochemistry, *In situ* hybridization, Insulin-like growth factor-I.

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The World Health Organization defines aneurysmal bone cyst (ABC) as an expanding osteolytic lesion consisting of blood-filled spaces of variable size that are separated by connective tissue septa containing trabeculae of bone or osteoid tissue and osteoclast

giant cells (1). Although the incidence of ABC is reported to be only 0.14 per 100,000 (2), since 1966, nearly 1000 articles have been published on this topic. The nature of ABC, however, has still remained unclear and controversial (3-8), even leading Vicenzi to the conclusion that none of the present etiopathogenic theories is based on objective and convincing data (9). Most recently, Sciot *et al.* reported chromosomal aberrations in ABC suggesting that at least some ABCs are true neoplasms (10).

Insulin-like growth factor-I (IGF-I) is a polypeptide of about 7.5 kDa that has been shown to mediate the effects of growth hormone on skeletal growth and to have a variety of anabolic effects on cells of connective tissue origin (11-13). IGF-I has been implicated in the autocrine or paracrine regulation of cell proliferation and differentiation (14). The liver is the main production center, but other sources of IGFs are available for IGF-responsive tissues via autocrine or paracrine pathways (11, 12). In some neoplastic diseases, IGF-I is produced by the tumor itself (15).

Overexpression of IGF-I and its receptor has been observed in osteosarcoma and soft-tissue sarcoma (16, 17), astrocytoma and meningioma (18), giant cell tumor of bone (19), and other tumors (15, 20, 21). Giant cell tumor of bone is sometimes strikingly similar to and occasionally indistinguishable from ABC (22). Therefore, the recent findings on IGF-I expression in giant cell tumor of bone (19) have been the stimulus to investigate the expression of IGF-I in ABC.

MATERIALS AND METHODS

Tissue Specimens

Nineteen samples of ABC were obtained from 19 individuals at surgery. Tissue sections were fixed in 4.5% formaldehyde and were paraffin embedded according to standard procedure. The tissue samples were analyzed histologically. As only a limited

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number of tissue sections was assigned to this study, the samples were randomly divided between the two laboratories involved for either immunohistochemistry or RNA *in situ* hybridization.

Eight cases occurred in females and 11 in males, with a median age at presentation of 14 years (range, 7–46 years). Tumor sites and patient characteristics are indicated in Tables 1 and 2.

Immunohistochemistry

Nine formalin-fixed, paraffin-embedded tissue sections of ABC were deparaffinized with xylene, rehydrated with graded ethanols, and rinsed in water. Immunoreactivity was enhanced by autoclaving the slides for 25 minutes and cooling the slides in the closed autoclave for an additional 40 minutes. The sections were then incubated with primary murine monoclonal antibodies for IGF-I (Upstate Biotechnology, NY) diluted in Tris buffer, pH 7.5, containing 2% horse serum, for 1 hour at room temperature. After washing with Tris buffer, the samples were incubated sequentially with biotinylated horse anti-mouse immunoglobulin diluted in horse serum for 30 minutes at room temperature, followed by avidin-biotin-complex-peroxidase (Vector Laboratories, Burlingame, CA). After rinsing in Tris buffer, peroxidase activity was detected with Fast Red (DAKO, Glostrup, Denmark). The tissue sections were counterstained with hematoxylin and covered with glycerol. All slides were evaluated by light microscopy. Monitoring of IGF-I-

expression in each sample (negative, weak positive, or strong positive) was performed by comparing the intensity of staining. Normal human bone tissue, present in other sections, served as a reliable internal negative control, and human kidney tissue, as a positive control tissue.

RNA *In Situ* Hybridization

Eight-micrometer-thick formalin-fixed and paraffin-embedded tissue sections were deparaffinized using xylene and ethanol according to standard procedure and air dried at room temperature. The sections were permeabilized for *in situ* hybridization by pepsin digestion with 2.5 mg/mL pepsin (Kreatech Diagnostics, Amsterdam, The Netherlands) in 0.1 M HCl for 30 minutes at 37° C. Slides were dehydrated with ethanol. For each slide, 50 ng of probes (366-bp-long digoxigenin-labeled cDNA fragments corresponding to human mRNA for IGF-Ia, positions 88–434) diluted in 20- μ l hybridization solution (Hybrisol IV, Oncor, Gaithersburg, MD) were applied. Slides were covered with a glass coverslip and sealed with rubber cement.

In order to ensure perfect permeabilization and denaturation of probes and targets, slides were placed in a metal box and heated for 5 minutes in an 80° C waterbath and then incubated overnight at 37° C. After hybridization, slides were washed three times in 50% formamide buffer for 10 minutes and two times in 2 \times SCC (2 \times SCC: 0.03 M sodium citrate

TABLE 1. Patient Characteristics and Results of Immunostaining for Insulin-Like Growth Factor-I in Aneurysmal Bone Cyst

Patient No.	Gender	Age (y)	Location	Diagnosis	Immunostaining	
					Stroma	Giant Cells
1	M	15	Tibia	ABC	++	+
2	M	10	Femur	ABC	++	++
3	M	7	Femur	ABC	++	++
4	M	21	Sacrum	ABC	++	++
5	F	22	Rib	ABC	++	+
6	M	13	Fibula	ABC	++	+
7	F	14	Ischium	ABC	++	++
8	F	19	Pubic	ABC solid	–	++
9	M	46	Ilium	ABC solid	++	+

+, weak expression; ++, strong expression; –, no expression detected.

TABLE 2. Patient Characteristics and Results of *In Situ* Hybridization with Antisense Oligonucleotides to Insulin-Like Growth Factor-I mRNA in Aneurysmal Bone Cyst

Patient No.	Gender	Age (y)	Location	Diagnosis	Detection Method	Intensity
10	F	11	Femur	ABC	Fluorescent	++
11	M	12	Femur	ABC	Fluorescent	++
12	F	10	Lumbar spine	ABC	Fluorescent	++
13	F	32	Radius	ABC	Fluorescent	+
14	M	11	Sacrum	ABC	Peroxidase	+
15	M	7	Humerus	ABC	Peroxidase	+
16	F	16	Femur	ABC	Peroxidase	++
17	F	17	Fibula	ABC	Alkaline phosphatase	++
18	M	32	Fibula	ABC solid	Alkaline phosphatase	++
19	M	12	Femur	ABC	Alkaline phosphatase	+

+, weak expression; ++, strong expression.

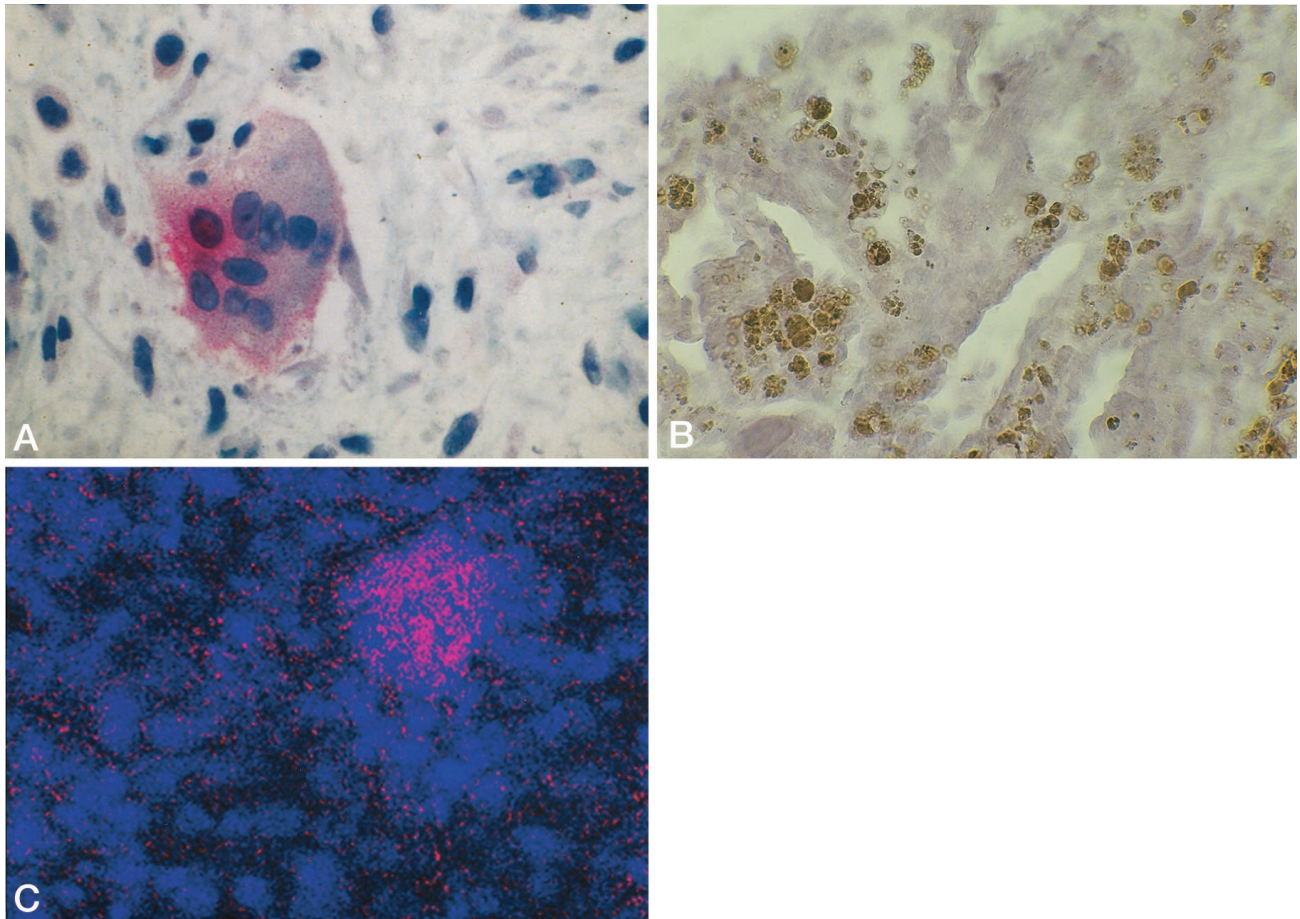


FIGURE 1. A, immunohistochemical analysis of IGF-I in ABC (Patient 9). A multinucleate giant cell is strongly positive for IGF-I (200 \times). B, *in situ* hybridization with digoxigenin-labeled antisense oligonucleotides to IGF-I mRNA (Patient 17). Probes were detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase. A multinucleate giant cell (on the left) and mononuclear stromal cells exhibit a strong signal (500 \times). C, fluorescent detection of digoxigenin-labeled IGF-I mRNA (Patient 10). Probes were detected with anti-digoxigenin antibodies conjugated to rhodamine. Cell nuclei are stained blue. The strong signal corresponds to a multinucleate giant cell (500 \times).

+ 0.3 M sodium chloride). After washing with phosphate buffered saline (PBS; three times, each for 10 minutes), riboprobes were detected by using fluorescent detection in four cases and colorimetric detection methods in six cases.

For fluorescent detection, slides were incubated with anti-digoxigenin antibodies conjugated to rhodamine (Roche Diagnostics, Mannheim, Germany) diluted 1:10 in PBS plus 1% blocking solution (Roche) for 30 minutes at 37 $^{\circ}$ C. Afterwards, slides were washed with PBS for 2 minutes and rinsed with water. Sections were incubated with 2- μ g/mL DAPI (Sigma, St. Louis, MO) in McIlvaine's buffer (0.1 M citric acid monohydrate + 0.2 M disodium hydrogen phosphate) for 20 minutes, mounted with 50% glycerol in PBS, and coverslipped.

For colorimetric detection, three sections were incubated with anti-digoxigenin antibodies conjugated to alkaline peroxidase (Roche) diluted 1:10 in PBS plus 1% blocking solution (Roche), and another three sections were incubated with anti-digoxigenin antibodies conjugated to horseradish peroxidase (*In Situ* Hybridization and Detection Kit, Kreatech) for 30

minutes at 37 $^{\circ}$ C. After washing with PBS and alkaline detection buffer (Roche), sections were incubated with NBT/BCIP (Roche) for detection of alkaline phosphatase. Horseradish peroxidase was detected by application of AEC solution (Kreatech) for up to 15 minutes at 37 $^{\circ}$ C. Then slides were washed with water and air dried. Sections were counterstained with methyl green-pyronine or giemsa, mounted with 50% glycerol in PBS, and coverslipped.

As a negative control for all three detection methods, sections incubated with plain hybridization solution, and smears of blood from healthy donors were used. Blood smears containing 50% B-cell blasts served as a positive control.

RESULTS

Detection of IGF-I in ABC by Immunohistochemistry

Immunoreactivity for IGF-I was found in all nine specimens of ABC tested (No. 1–9). No difference in the level of IGF-I was found between seven cystic

ABCs (No. 1–7) and two solid ABCs (No. 8 and 9). Mononuclear stromal cells were strongly positive for IGF-I in eight of nine samples. Multinucleate giant cells were strongly positive in five of nine samples, and a weak expression was found in four of nine samples (Fig. 1A). In contrast, in none of the specimens did any normal human bone tissue show significant immunostaining for IGF-I.

In Situ Hybridization

To determine whether elevated levels of IGF-I in ABC, as revealed by immunohistochemistry, occur due to overexpression of IGF-I in ABC or due to increased binding of exogenous IGF-I, *in situ* hybridization was performed on ten specimens of ABC with an antisense IGF-Ia digoxigenin-labeled riboprobe. Three different detection methods were applied. All specimens tested showed positive staining for IGF-I mRNA. Four of ten samples (No. 13–15 and 19) displayed weak staining; in six of ten samples (nos. 10–12 and 16–18), staining for IGF-I mRNA was strong. In all specimens tested, IGF-I transcripts were located in multinucleate giant cells but also in mononuclear stromal cells (Fig. 1B+1C). In contrast, staining was absent in the negative controls, thus excluding false positive results as obtained by autoimmunofluorescence or unspecific enzymatic activity that might have resulted from, for instance, hemosiderin.

DISCUSSION

The results of this study demonstrate that IGF-I is overexpressed in ABC. Together with reports on the growth-promoting activity of IGF-I (13) and on the expression of IGF-I in many bone tumors (16, 17, 19), including giant cell tumors (19), the results of this study indicate that locally produced IGF-I might play a role in the development and growth and of ABC. Furthermore, multinucleate giant cells, which have been shown to synthesize IGF-I in giant cell tumors (19), might be of importance in that process.

An important differential diagnosis of ABC is giant cell tumor of bone (22). Microscopically, it is sometimes impossible to tell the two apart (22). Comparing the findings of IGF-I-expression in ABC with the previously reported findings in giant cell tumor (19), there seems to be no major difference in IGF-I-overexpression. Therefore, this aspect is not helpful for a practical diagnostic application.

As IGF-I is directly involved in fracture healing (23), the history of all patients was reviewed with special attention to a possible pathologic fracture caused by the osteolytic defect. Four of the patients (No. 11, 13, 15, and 19) had presented with this complication. However, *in situ* hybridization

showed no difference between nonfractured and fractured ABCs.

Several conditions with elevated IGF-I serum levels, such as pregnancy (24, 25), trauma (26), and the adrenogenital syndrome (27), display a coincidence with ABC (28–38). Furthermore, ABC has a peak incidence in the first two decades (2), in which IGF-I serum levels are reported to be high (39–41). Also, for Van Buchem's disease, which is similar to acromegaly (42, 43), a disorder with elevated IGF-I serum levels (44, 45), a coincidence with ABC has been described (37). The involvement of IGF-I in the pathogenesis of ABC might explain the coincidence of these conditions with ABC.

In conclusion, the data presented in this study demonstrate that IGF-I is overexpressed in ABC, indicating a possible role of the IGF-I-system in the pathogenesis and growth of this lesion. Other curious phenomena like the five reported familial cases of ABC (9, 46–49) and the several congenital cases (50–52) could be better understood by searching for an underlying, possibly genetic defect in the growth hormone/IGF-I system. However, the exact mechanisms of the growth of ABC, the role of multinucleate giant cells, and the true nature of this entity remain unclear.

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