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RESEARCH

Does Lactoferrin Behave as an Immunohistochemical Oncofetal Marker in Bone and Cartilage Human Neoplasms?

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Abstract By immunohistochemistry, lactoferrin (LF) has been extensively investigated in human neoplastic tissues; moreover, LF is able to promote bone growth in a murine model. Until now, no systematic studies on human osteocartilagineous fetal samples have been performed in comparison to corresponding neoplastic specimens to verify if LF may represent an oncofetal marker in this field of pathology. By a monoclonal antibody (clone 1A1; Biodesign International; w.d. 1:75) the distribution pattern of LF in bones of 25 human fetal tissues (8–34 gestation weeks), 10 adults (47–82 years) and 30 cartilage as well as 27 bone tumours (9–76 years) was analyzed. LF was encountered in 23/57 cases of osteocartilagineous tumors and namely in 10/10 giant cell tumours, 5/7 osteoid osteomas, 3/3 chondroblastomas, 3/3 chondromyxoid fibromas, 1/1 myeloma, 1/1 adamantinoma. No LF immunoexpression was detected in osteosarcomas, chondrosarcomas, ossifying fibromas, osteochondroma and enchondromas. In embryo-fetal tissues, LF immunoreactivity was localized in mesenchymal cells as well as in chondroblasts at the 8th gestational week and in immature osteocytes and osteoblasts up to the 18th gestation week, with a considerable decrease by the 24th week. No LF expression was found in any bone district since the 30th and up to the 34th week of gestation as well as in corresponding adult samples. Our findings indicate a role for LF as a bone growth regulator in the early phases of the human endochondral ossification, although the hypothesis of LF as oncofetal marker appears questionable in bone tumours.

Keywords Bone tumours · Fetal tissue · Immunohistochemistry · Lactoferrin · Oncofetal antigens

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Introduction

The so-called oncofetal or oncodevelopmental antigens are those commonly present at high concentration in the embryo and fetus during early development of life or in the placenta, but undetectable or present at low concentration in the adult, appearing only when cancer occurs [1–3]. These antigens, primarily glycoprotein in nature, are the products of one or more genes that are normally expressed only during fetal development and then repressed in the adult life [3]. Their production in adults is a result of the activation of the controlled genes by a yet unknown mechanism in association with cancer, so these antigens have been considered as tumor markers [3–5]. In detail, an oncofetal marker is a tumor marker produced by tumor tissue and by fetal tissue of the same type as the tumor, but not by normal adult tissue from which the tumor arises [6].

The most common oncofetal tumor marker are alpha-fetoprotein (AFP), human chorionic gonadotropin (hCG), carcinoembryonic antigen (CEA), cancer antigen 125 (CA 125), tissue polypeptide-specific antigen (TPS), carbohydrate antigen 19-9 (CA 19-9) and prostate–specific antigen (PSA) [3]. The presence of considerable concentrations of AFP, hCG, CEA, CA 125, TPS, CA 19-9 and PSA in the

amniotic fluid during pregnancy has been attributed to their involvement in biological functions associated with fetal development, differentiation and maturation [3].

It has been demonstrated that lactoferrin (LF), a 80 kDa single-chain major iron-binding protein, is expressed during murine embryonic development [7]. Moreover, it has been demonstrated that LF stimulates the proliferation and differentiation of rat primary osteoblasts in vitro together with the synthesis and mineralization of the bone matrix [8–10]. However, the expression pattern of LF, if any, during embryo or foetus development in humans has not been investigated to date. To gain further insights into the function of LF as an oncofoetal antigen, we have investigated the expression of this glycoprotein in osteocartilagineous humans tissues during early and late gestation, using a monoclonal antibody against human lactoferrin, in comparison to a cohort of bone and cartilagineous human neoplastic samples.

Materials and Methods

Samples of unaffected human cartilage and long and flat bone tissue, taken at autopsy from 25 foetuses (ranging between 8 to 34 weeks of gestation) and ten adults (range from 47 to 82 years; the men age was 68 years), as well as 57 benign and malignant neoplastic samples of bone and cartilage tumours, derived through curettage or surgery from an equal number of patients (33 males, 24 females; age range: 9–76 years; mean age 36,77 years), were submitted to the immunohistochemical procedures for LF. All specimens were obtained with consent from the Local Ethics Committee.

All samples were fixed in 10% neutral formalin for 24 h at room temperature (RT) and then embedded in paraffin at 56°C. The bone specimens were decalcified using formic acid 5% or EDTA 5%, pH 7.4, for a period not longer than 48 h, depending on the size of mineralised samples. From each tissue block, 4 μm-thick sections were stained with haematoxylin/eosin for the microscopic evaluation, with Perls'Prussian blue ferrocyanide and von Kossa methods. Parallel sections were cut and mounted on silane-coated glasses, then dewaxed in xylene and rehydrated in graded ethanols. Antigen retrieval was performed before adding primary antibody by heating slides placed in 0.01 M citrate buffer, pH 6.0, in a microwave oven for three cycles × 5 min. For the immunohistochemical study, sections were treated in a moist chamber: (1) with 0.1% H_2O_2 in methanol to block the intrinsic peroxidase activity (30 min at RT); (2) with normal sheep serum to prevent unspecific adherence of serum proteins; (3) with the monoclonal primary antibody against anti-human LF (clone 1A1; Biodesign International, Saco, ME; w.d. 1:75; 60 min at RT); (4) with sheep anti-mouse immunoglobulin antiserum (Behring Institute, Marburg, Germany; w.d. 1:25; 30 min at RT); (5) with mouse anti-horseradish peroxidase-antiperoxidase complexes (Dako Cytomation, Copenaghen, Denmark w.d. 1:25; 30 min at RT). For the demonstration of peroxidase activity, the sections were incubated in darkness for 10 min with 3-3′ diaminobenzidine tetra hydrochloride (Sigma Chemical Co., St Louis, MO), in the amount of 100 mg in 200 ml 0.03% hydrogen peroxide in phosphate-buffered saline (PBS). The nuclear counterstain was performed by Mayer's haemalum. Renal tubular structures within normal kidney samples as well as portions of parotid gland were utilised as additional positive controls [11]. Moreover, LF immunoreactivity demonstrated in granules of polymorphonuclear neutrophils was utilised as positive control. Finally, in order to test the inter-run variability of LF staining, the same LF-positive parotid sample was utilised in every run. To test the specificity of LF immunostaining in order to deny the possibility of non-specific reaction, serial sections of each bone or cartilage specimen were tested by replacing the specific antiserum by either PBS, normal rabbit serum or absorbing with excess of purified human LF from human liver and spleen (Sigma Chemical Co.) as well as with pre-absorbed primary antibody: the results obtained were negative.

Immunostained sections were estimated by light microscopy using a ×20 and ×40 objective lens and ×10eyepiece; the assessment of LF immunostained sections was performed on a consensus basis by two pathologists (A.I. and G.T.) using a double-headed microscope.

Results

Routinely stained haematoxylin-eosin sections exhibited a good morphology, confirming the histopathological diagnosis in all neoplastic cases. With the Perls' method, an occasional positive granular appearance, attributable to hemosiderin granules was found in the neoplastic stroma or in the cytoplasm of histiocytes. The cores of dark blue calcified cartilage representing the newly formed bone in fetuses were revealed by von Kossa method. Analytical data concerning clinicopathologic characteristics of fetuses as well as neoplastic samples are reported in Tables 1 and 2 respectively.

With reference to the normal foetal samples, LF immunolabelling was mainly localized at the cytoplasm and sometimes at the nucleus of stained elements. At the 8th week of gestation LF immunoreactivity was found in the mesenchymal cells (MSCs) forming the periosteum as well as in chondroblasts within all the calcified cartilage samples (Fig. 1a). These latter elements exhibited a weak to moderate LF staining, while MSCs showed a strong

Table 1 Analytical findings of 25 foetuses examined

Case	Sex	Age of Gestation	Bone site
1	M	8	Rib
2	M	8	Vertebrae
3	F	9	Humerus
4	M	9	Rib
5	M	10	Femur
6	F	10	Humerus
7	M	11	Vertebrae
8	M	11	Rib
9	F	12	Femur
10	M	13	Vertebrae
11	F	14	Humerus
12	M	15	Femur
13	F	16	Femur
14	F	17	Humerus
15	M	17	Femur
16	F	18	Femur
17	M	20	Rib
18	M	21	Vertebrae
19	F	22	Humerus
20	M	24	Femur
21	M	26	Rib
22	M	29	Femur
23	F	30	Rib
24	M	32	Humerus
25	F	34	Femur

immunoexpression (Fig. 1a). At the 12th week, a strong LF immunoexpression was found in immatures osteocytes and osteoblasts enclosed or lining the spicule (Fig. 1b) as well as in chondroblasts of peripheral calcified cartilage. At the 18th weeek, a constant increase in the number of LF positive osteoblasts inside the bony matrix was noted (Fig. 1b), but a considerably decrease of staining was encountered in these cells by the 24th and up to the 29th week of gestation. No immunostaining was seen in the mature chondrocytes beetween 18th and 29th gestational weeks. In addition, no LF expression was found in the osteo-cartilagineous foetal samples since the 30th week and up to the 34th, similarly to the adult bone tissue samples. The osteoclast lineage was always unstained by LF antibody.

On neoplastic specimens, LF immunoreactivity was encountered in 23/57 cases of osteo-cartilagineous tumors and namely in 10/10 giant cell tumours (GCTs), 5/7 osteoid osteomas (OO), 3/3 chondroblastomas (CBL), 3/3 chondro-myxoid fibromas (CMF), 1/1 myeloma, 1/1 adamantinoma. In the GTCs, LF immunoreactivity was seen in the neoplastic stromal cells as well as in multinuclear giant cells (MNGC). In

Table 2 Clinico-pathological data referred to 57 analyzed osteocarti-lagineous tumors

Case	Sex	Age	Localization	Histotype
1	M	23	Humerus	ECH
2	F	19	Tibia	ECH
3	M	31	Hand	ECH
4	M	23	Femur	ECH
5	M	46	Femur	ECH
6	F	50	Hand	ECH
7	M	48	Hand	ECH
8	F	50	Hand	ECH
9	F	28	Femur	ECH
10	M	44	Hand	ECH
11	F	36	Femur	ECH
12	F	72	Sternum	ECH
13	F	49	Pelvis	ECH
14	M	31	Femur	ECH
15	M	40	Hand	ECH
16	M	26	Femur	OCH
17	M	25	Hand	OCH
18	M	40	Rib	OCH
19	M	41	Hand	OCH
20	F	23	Hand	OCH
21	M	30	Humerus	OCH
22	M	37	Jaw	CS
23	F	57	Humerus	CS
24	M	40	Humerus	CS
25	M	9	Femur	CBL
26	F	17	Humerus	CBL
27	M	28	Humerus	CBL
28	F	25	Tibia	CMF
29	F	28	Tibia	CMF
30	M	21	Femur	CMF
31	M	40	Tibia	OO
32	M	37	Fibula	OO
33	F	25	Femur	OO
34	M	59	Fibula	OO
35	M	32	Fibula	OO
36	F	17	Femur	OO
37	M	14	Fibula	OO
38	M	48	Hand	GCT
39	F	50	Femur	GCT
40	M	43	Hand	GCT
41	F	28	Hand	GCT
42	M	33	Femur	GCT
43	F	58	Hand	GCT
44	F	63	Pelvis	GCT
45	M	28	Tibia	GCT
46	F	45	Hand	GCT
47	M	33	Femur	GCT
48	M	28	Femur	OS

Table 2 (continued)

Case	Sex	Age	Localization	Histotype
49	F	72	Femur	OS
50	M	25	Femur	OF
51	M	21	Femur	OF
52	F	27	Hand	OF
53	F	25	Tibia	OF
54	M	76	Femur	OF
55	F	35	Femur	OF
56	F	69	Femur	Myeloma
57	M	28	Tibia	Adamantinoma

ECH chondroma, *OCH* osteochondroma, *CS* chondrosarcoma, *CBL* chondroblastoma, *CMF* chondromyxoid fibroma, *OO* osteoid osteoma, *GCT* giant cell tumor, *OS* osteosarcoma, *OF* ossifying fibroma

the osteoid osteomas, LF immunolocalization was encountered in the mature and immature osteoblasts that lined the bone spicules. Moreover, LF reactivity was localized in the chondroblast-like cells and in multinucleated giant-cells present in all chondroblastomas (Fig. 2a) and it was also present in the spindle cells within the stroma of the chondromyxoid fibromas. In the case of adamantinoma, the positivity was located in the epithelioid cells. Finally, the neoplastic elements within the myeloma case displayed a positive LF immunoreaction. No LF immunoexpression was detected in chondrosarcomas (CS) (Fig. 2b), osteosarcomas (OS) (Fig. 2c), ossifying fibromas (OF), osteochondroma (OCH) and enchondromas (ECH). Data on the site of occurrence of these tumors were available and reported in Table 2.

LF immunolocalization was evident in all ductular/acinar structures of the parotid as well as kidney tubular structures, utilized as positive controls.

Discussion

Lactoferrin was originally isolated from milk but its presence has been also demonstrated in many human exocrine secretions [12], although its concentration widely varies between different species and at different stages of lactation [13]. Several physiological roles have been attributed to LF, namely regulation of iron homeostasis, host defence by antibacterial and antiviral activities, regulation of cellular growth and differentiation [14–16]. On the other hand, it has been shown that LF inhibits the growth of tumours and confers protection towards metastasis, suggesting that LF may play a role in the defense against tumorigenesis [17–19]. These latter findings have suggested LF's great potential therapeutic use in cancer disease prevention and/or treatment, namely as a chemopreventive agent [16].

LF expression has been extensively investigated through immunohistochemistry in human neoplastic tissues, such as adenocarcinomas of the parotid gland [20], prostatic carcinomas [21], breast carcinomas [17, 22, 23], thyroid tumours [24–26], gastric adenomas and carcinomas [27], colorectal adenomas and carcinomas [28], gallbladder carcinomas [29], astrocytomas and multiforme glioblastomas [30], pigmented skin lesions [31], endometrial [32], renal cell carcinomas [11] and recently also in bone and cartilage tumours [33, 34].

In comparison to a cohort of osteocartilagineous tumours, we have hereby analyzed the LF immunoexpression in normal human foetal and adult bone tissue in order to evaluate its possible role as an oncofetal marker. In detail, LF immunoreactivity was encountered both in the nucleus and the cytoplasm of the osteocartilaginous tissues from human embryos and foetuses from 8th until 29th gestation gestational

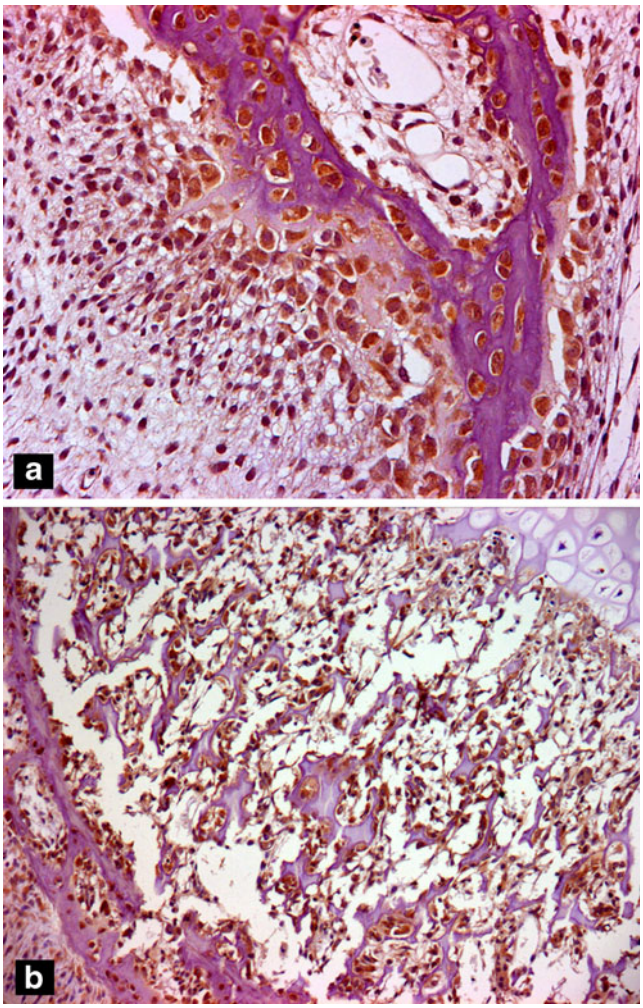

Fig. 1 LF immunopositivity was encountered in chondroblasts and mesenchymal cells forming the periosteum (**a** 10th gestation week, ×200) as well as in immatures osteocytes and in osteoblasts (**b** 22nd gestation week, ×200) (Immunoperoxidase; Mayer's haemalum counterstain)

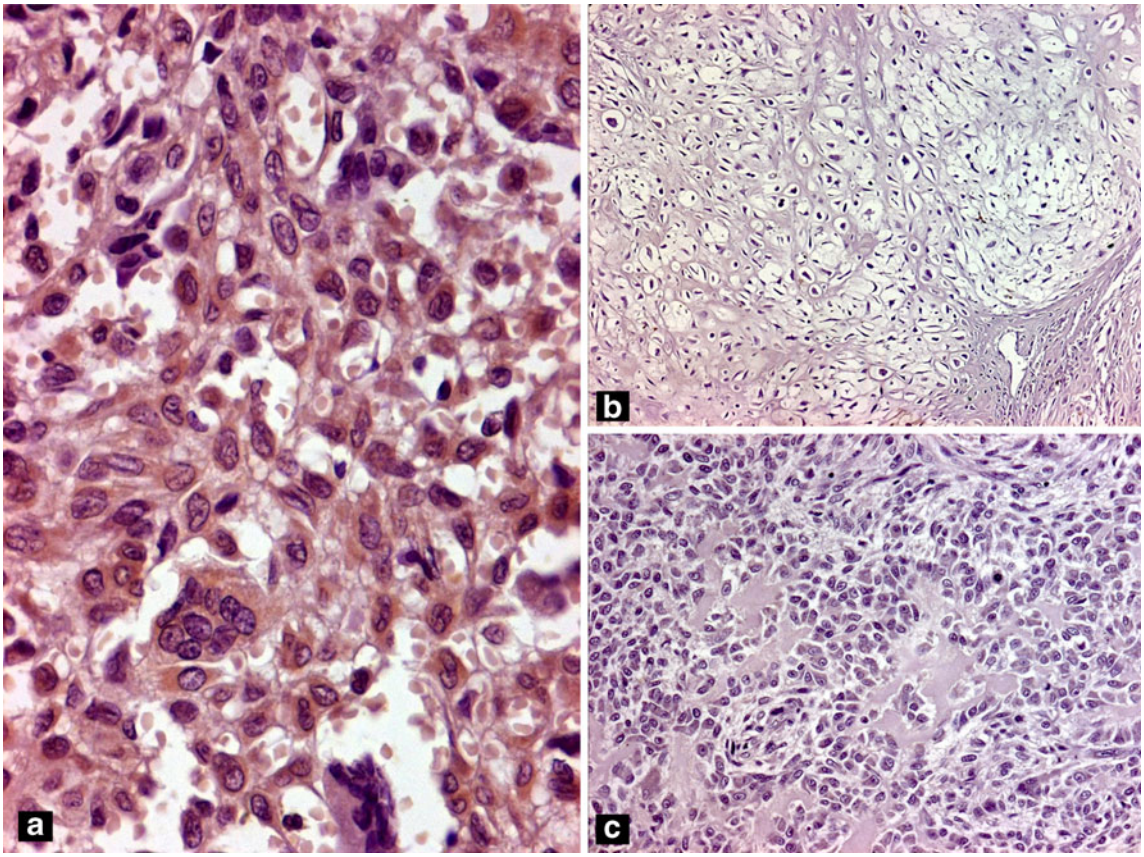

Fig. 2 An evident LF reactivity was localized in chondroblast-like cells as well as in multinucleated giant-cells of chondroblastomas (**a** × 800); by contrast, no LF immunostaining was found in neoplastic elements of chondrosarcomas (**b** ×400) and osteosarcomas (**c** ×400) (Immunoperoxidase; Mayer's haemalum counterstain)

weeks as well as in 23/57 neoplastic samples. The site of LF immunoreactivity was not surprising, because this secretory protein has been already detected in the nucleus, mainly in the nucleoli, and it has been thought to be involved in ribosomal biogenesis [35, 36].

In the neoplastic group, the LF immunostaining was confined to cases of GCT, OO, CBL and CMF; no immunoreactivity was found in other kinds of tumours, such as in osteosarcomas and chondrosarcomas. On the other hand, LF immunoexpression was not found in the bone and cartilagineous embryo-foetal tissues after the 29th week of gestation, similarly to the osteo-cartilagineous samples from adults. The pattern of LF expression in foetal tissues togheter with its absence at the end of pregnancy and at term may allow to include LF in the class of oncofetal proteins associated with foetal development and differentiation. Neverthless, LF appearance in tumours is independent from benign or malignant characteristics; in fact, for example, the most aggressive bone tumours such as OS and CS were always unreactive for LF, in contrast to that expected in malignancy, if LF behaves as a tumour oncofetal marker. However previous studies in other malingnancies, such as tumours of the parotid, prostate, breast, thyroid, gallbladder, stomach, brain and endometrium [20–22, 24, 26, 27, 29, 30, 32], have shown that LF was mainly appreciable in differentiated tumours, but only seldom in anaplastic ones. Therefore, taking into consideration the heterogeneity of neoplastic sites, it has been hypothesized a role for LF as a marker of cellular differentiation [29, 30]. Therefore, the presence of LF in well differentiated tumours may be related to its capability to bind iron, an essential nutrient for cells that are growing and dividing [30–32].

Interestingly, we have documented the LF presence in stromal cells of GTCs, that are known to be derived from mesenchymal stem cells (MSCs) [37–39], similarly to foetal osteoblasts and chondroblasts, which are multipotent cells that reside in the bone marrow [40, 41]. However, we have encountered LF immunoexpression in foetal osteblasts and chondroblasts as well as in perichondrial MSCs, suggesting thus an histogenetic link. In addition, the immunopositive LF pattern by us reported in CBL and CMF could be attributed to their common origin from mesenchymal stem cells (MSC), similarly to that elsewhere suggested in order to explain the neoplastic histogenesis of these tumours [42]. Furthermore, a role of LF in the

mechanism of differentiation of MSCs into osteoblastic and chondroblastic lineages has been recently proposed [43]; this latter suggestion, togheter with our observations in foetal osteocartilagineous tissues, may substantiate an important action of LF as a bone growth regulator/factor in the early phases of the endochondral ossification.

Finally, our findings are unable to demonstrate a possible oncofetal mechanism for LF in osteocartilagineous tumours since its distribution is quite heterogenous in tumours, even if LF is largerly present in the corresponding foetal tissues. The multifunctional histogenetic and differentiative role of LF in tumours appears more acceptable, although future additional investigations may clarify its applicative perspectives.

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