Available online at www.sciencedirect.com
SCIENCE DIRECT
Analytical Biochemistry 354 (2006) 205–212
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

A comparison of methods for the analysis of low abundance proteins in desmoid tumor cells

L. Picariello [a], S. Carbonell Sala [b], V. Martineti [b], A. Gozzini [b], P. Aragona [b], I. Tognarini [b], M. Paglierani [c], G. Nesi [c], M.L. Brandi [b,d,*], F. Tonelli [a]

[a] *Department of Clinical Physiopathology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy*
[b] *Department of Internal Medicine, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy*
[c] *Department of Human Pathology, University of Florence, Viale Morgagni 85, 50139 Florence, Italy*
[d] *Department of DeGene Spin-off, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy*

Received 20 December 2005
Available online 3 May 2006

Abstract

The desmoids are a group of rare clinically diverse, deep-seated fibrous neoplasms. The exact etiology is unknown, but several factors are considered to be positively correlated with their development and growth, i.e., genetic and hormonal factors and trauma. These tumors may be sporadic or associated with a genetic disease such as familial adenomatous polyposis (FAP). Devoid of metastatic potential, they tend to form large, infiltrative masses which, if not completely excised, recur repeatedly. Although surgery is widely accepted as the first-line treatment for extra-abdominal and abdominal wall desmoids, a proportion of cases are successfully palliated with either estrogen antagonists (tamoxifen, toremifene, and raloxifene) or nonsteroidal anti-inflammatory drugs. We describe and compare four methods for evaluating the expression of estrogen receptors α/β and COX-1 and COX-2 in desmoid tumor-derived cells and tissues: immunocytochemistry, immunohistochemistry, RT-PCR, and two-color Western blot detection with the Odyssey infrared imaging system. Through this comparative analysis, Western blot with Odyssey was recognized as the best method to analyze the expression particularly of low expressed proteins in desmoid-derived cells. The use of a specific and reliable assessment method becomes fundamental in the evaluation of the presence and modulation of proteins which are important but weakly expressed in these rare tumors.

Keywords: Desmoid tumors; Protein analysis; Estrogen receptors; Infrared imaging system

Desmoid tumors are a group of clinically diverse, deep-seated fibrous neoplasms. Although we do not know the exact etiology of these tumors, several factors are considered to be positively correlated with the development and growth of desmoids: trauma and genetic and hormonal factors, such as the endogenous estrogen levels in pregnancy [1–3].

They account for less than 0.1% of all tumors [4] with an annual incidence of 2–4 per million [1]. Desmoids can be divided into two main biologic groups: sporadic and associated with familial adenomatous polyposis (FAP),[1] a genetic disease caused by different mutations in the tumor-suppressor APC gene. Anatomically, there are three main subsets: extra-abdominal (60% of cases), abdominal (25%), and intra-abdominal (15%). In all of these groups, the peak of incidence is in the second to fourth decades, although the overall age range is wide [5]. Approximately 70–90% of cases of desmoid of the abdominal wall occur in women during the reproductive age [1] and quite frequently during or soon after pregnancy [6]. Irrespective of location, all tend to

* Corresponding author. Fax: +39 55 4296585.
E-mail address: m.brandi@dmi.unifi.it (M.L. Brandi).

[1] *Abbreviations used:* FAP, familial adenomatous polyposis; ERs, estrogen receptors; ICC, immunocytochemistry; IHC, immunohistochemistry; RT-PCR, reverse transcriptase–polymerase chain reaction; WB, two-color Western blot detection with the Odyssey infrared imaging system; FCS, fetal calf serum; CS-FCS, charcoal-stripped FCS; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

0003-2697/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2006.03.047

form large, infiltrative masses which, if not widely and clearly excised, recur repeatedly [7–9]. These lesions are, however, free from metastatic potential.

Histologically, desmoid tumors are composed of fibroblasts and myofibroblasts. Cellular amount and mitotic activity may vary considerably both within each tumor and between separate ones. The overall growth pattern is that of broad, elongated fascicles, while the stroma is collagenous and contains a varying number of blood vessels [5].

Treatment of desmoid tumors is difficult because of the high rate of recurrence after surgical removal. Excision is often incomplete because of massive infiltration of vital structures, and recurrence is at times destructive and uncontrolled [10,11]. The higher incidence of desmoids in females during the reproductive age and the fact that desmoid tumor-derived tissues and cells express estrogen receptors (ERs) [12–15] suggest that estrogens play a role in the etiopathology of this neoplasm. Paradoxically, there is little or no correlation between the presence of ERs in tumor cells and the response to hormonal therapy [16]. In fact, how the estrogenic molecules modulate tumor size is still unknown, but evidence exists for autocrine and paracrine mechanisms through which the estrogen receptor modulators, triphenylethylenes, could modulate the production of growth factors by desmoid tumor cells and inflammatory tumor-associated cells [17,18].

Tumor regression has also been reported in patients treated with non steroidal anti-inflammatory drugs, presumably because they interfere with the prostaglandin system, blocking the two isozymes of cyclooxygenase, COX-1 and COX-2 [19–21]. Because of this, although surgery is widely accepted as the first-line treatment for extra-abdominal and abdominal wall desmoids, a proportion of cases are successfully palliated with either estrogen antagonists—such as tamoxifen, toremifene, and raloxifene—or non steroidal anti-inflammatory drugs [20,22–24].

Desmoid tumors are rare tumors, with small series in the literature detailing the clinical setting and therapy of the disease [7], and little has been reported regarding both the development of desmoid cell cultures [12,13,17,25] and the optimization of suitable methods for the study of protein expression in these cells and tissues.

Here, we describe and compare four methods of evaluating the expression of either ERα and β or COX-1 and COX-2 in desmoid tumor tissues and derived cells: immunocytochemistry (ICC), immunohistochemistry (IHC), RT-PCR, and two-color Western blot detection with the Odyssey infrared imaging system (WB) (LI-COR, Biosciences, NE, USA) a powerful method for the in vitro analysis of the expression of low protein levels.

Materials and methods

Cell cultures

As a desmoid tumor continuous cell line is not commercially available, all the studies were performed using desmoid tumor primary cell cultures and long-term cultures between the first and the fifth passage. Primary cell cultures were obtained from desmoid tumor biopsy specimens taken at surgery from seven different patients. The patients gave their written consent and the local Institutional Review approved the study.

The cell growth medium (Complete medium) used in our study was Ham's F-12 Coon's modification (Sigma, St. Louis, MO), supplemented with 10% fetal calf serum (FCS) (Cambrex, NJ, USA), 1% non-essential amino acids (Sigma), 100 U/ml penicillin, and 100 U/ml streptomycin. Tissue fragments were collected in sterile tubes with Complete medium at room temperature and rapidly transferred to the laboratory, where the tissues were placed in a petri dish containing a small volume of Complete medium, and dissected into minute fragments (1–4 mm^2) with the use of a disposable scalpel and forceps. Each of these fragments was moved to a new petri dish without medium (about 10 fragments per dish) and covered with a coverslip to ensure that the tissue adhered to the bottom of the plate. Subsequently the Complete medium was added and the plates were transferred to an incubator for cell culture and maintained in a humidified 5% CO_2 atmosphere at 37 °C. The cells originated from the fragments were cultured until confluence and then detached with trypsin/ethylenediaminetetraacetic acid solution; subsequently the fragments were detached from the bottom of the plate and transferred to another one. The process described above was repeated. To investigate ERs expression, the cells were cultured in RPMI without phenol red and supplemented with 10% charcoal-stripped FCS (CS-FCS) (Sigma). Part of the tissue fragments obtained at surgery were collected in cryogenic vials containing RNA *later* (Sigma) a stabilization solution for tissue to preserve them at −20 °C for expression studies.

Immunocytochemistry

For ICC of ERs, subconfluent desmoid cells were cultured on sterile microscope slides, (Superfrost Plus, Menzel-Glaser, Germany) in RPMI without phenol red and without CS-FCS for 36 h. Then the slides were fixed in 95% ethanol. To evaluate COXs expression, subconfluent desmoid cells were cultured on sterile microscope slides in Complete medium. The hybridization with primary and secondary antibodies and the staining of the slides are described below.

Immunohistochemistry

Specimens were obtained by surgical resection in all cases and fixed in 10% buffered formalin before being processed in paraffin. In order to confirm the histological diagnosis a pathologist reviewed hematoxylin-eosin-stained sections from each specimen.

For IHC a representative 4-μm section for each lesion was selected. All sections were deparaffinized in Bio-Clear (Bio-Optica, Milano, Italy) and rehydrated using graded ethanol. To block endogenous peroxidase activity, the