Demonstration of Myofibroblasts Using a Novel Murine Monoclonal Antibody, 3C2G10.7 Raised from Proliferating Cells in Dupuytren's Disease

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Introduction

The concept of the existence of a type of connective tissue cell that has properties of fibroblasts and smooth muscle cells is not new. The presence of such cells in chicken and rat aorta [7,21,25] was initially demonstrated, using the electron microscope, some 30 years ago. These cells have subsequently been described in tissues including the capsules of the adrenal gland [32] and testicle [12] and granulation tissue of rat [9] and human [34]. The dual functions as fibroblasts and smooth muscle cells have been studied by in vivo autoradiographic techniques [26,31], in vitro contractile response [23], and comparative cell modulation and growth dynamics [41]. In 1974, the term "myofibroblast" was coined [34], the ultrastructural features of this differentiated fibroblast defined, and the cells purported to be the basic locomotive units in wound contraction. The discovery of the myofibroblast was soon followed by a multitude of ultrastructural studies of the cells in a great number of pathologic fibrocontractive conditions and fibrous neoplasms [22]. Significantly, fibrogenesis in aortic atherosclerosis [15,16,28], hypertrophic scar, [3,4] and Dupuytren's disease [5,8,19] have been attributed to the presence of myofibroblasts, and their uniform presence in tumors, e.g., atypical fibroxanthoma of skin [42,43] and malignantfibrous histiocytoma [6,40], raises important questions concerning the histogenesis of these fibrohistiocytic tumors.
The use of electron microscope to study the myofibroblasts is often hindered by its very restrictive sample size. Apart from documenting the presence of such a cell, it is difficults to appreciate the spatial relationship between the myofibroblasts and other cell types within the tissue. Similarly, the use of light microscope is not without its problems. Despite the distinctive ultrastructural appearance, the nuclear irregularities are hard to appreciate by light microscopy. The cytoplasmic microfilaments and special cell to cell and cell to stroma connections are beyond the normal light microscopic resolution. In fact, myofibroblasts are probably underdetected because of their rather nondescript light microscopic appearance, and they are often confused with fibroblasts. It is of interest to note, for example, that myofibroblasts are seldom mentioned in surgical pathology reports despite their ubiquitous presence in many pathologic conditions.

Confronted by the difficulties with the traditional morphologic approach in our studies of myofibroblasts and Dupuytren's disease, we embarked upon raising a monoclonal antibody that could be used immunohistochemically on routine paraffin sections to differentiate myofibroblasts from fibroblasts and other connective tissue cells. The murine monoclonal antibody that was raised, designated as clone 3C2G10.7, appears to fulfil the requirements as a specific marker for myofibroblasts.

Experimental Design

A monoclonal antibody which identifies myofibroblasts was produced from a hydridoma created by the fusion of mouse plasmacytoma cells and spleen cells of mice immunized with a cell suspension of profilerative stage Dupuytren's tissue [24,30] rich in myofibroblasts. The functional activity of the antibody was determined immunohistochemically by the avidin – biotin peroxidase technique [26] using fresh-frozen and formalin-fixed tissue.

Methods

Antibody Preparation

Specimens of palmar fascia were obtained at the time of surgery and the sample was dissected free of surrounding fibrofatty tissue in the operating room. Active stage of the disease in nodular tissue was confirmed by frozen tissue section stained with hematoxylin. Disaggregation of the remaining nodular tissue, which has been placed in sterile Hank's solution (Gibco, Grand Island, New York) for transport to the laboratory, was achieved by first cutting the sample into fine pieces with scissors. A single cell suspension was attained by gently agitating the pieces at 37°C in a solution of RPMI 1640 with 25 m*M* hepes buffer 10% fetal calf serum (Gibco), collagenase type 1A (10 mg/ml) (Sigma, St. Louis, Missouri) and DNase (1.5 mg/ml) (Sigma) for about 4 h or until no clumps of tissue were visible. The cells were washed and pellected twice in a solution of RPMI 1640 and 20% fetal calf serum in order to deactivate the enzymatic activity. Aliquoits of 1×10^7 cells/ml were frozen in fetal calf serum and 5% dimethyl sulfoxide (DMSO) and stored at −80°C until required. Six week old female Balb/c mice (Harlan-Spagrue-Dawley, Indiana) were primed with 1×10^7 thawed, washed cells suspended in an emulsion of 0.1 ml RPMI 1640 medium and 0.1 ml Freund's complete adjuvant by two subcutaneous injections in the back. The mice were subsequently boosted intraperitoneally with the same number of cells suspended in RPMI 1640 and Freund's incomplete adjuvent 9 days later. Three days after the last injection, using the hydridoma technique previously described [29], the mice spleen cells were mixed with the plasmacytoma cell line 315.43 in a 5:1 ratio and fusion initiated using 40% polyethylene glycol (PEG) (Sigma). The fused cells were

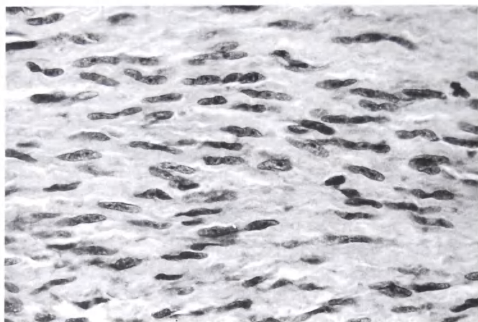
cultured in HAT restriction media supplemented with whole Balb/c mouse blood in 24-well plates seeded at 2×10^5 cells/well until the hydridomas were macroscopically visible. Supernantants from all wells were initially screened at 10–14 days postfusion and selected positive hydridomas were cloned and subcloned by the method of limiting dilution.
The immunoglobulin class was identified by using an alkaline phosphatase immunoassay kit (Innogenetics N.V., Belgium). In this procedure, strips, coated with separate bands of rat monoclonal antibodies against different mouse subclasses, were incubated with the supernant. The murine antibody in the sample reacted with the corresponding isotype band and was detected using rate anti-mouse Ig kappa, labeled with alkaline phosphatase. A dark band, proportional to the amount of specific antibody in the sample, was revealed after incubation with the substrate (5-bromo-4-elboro-3 indoyl phosphate in dimethyl formamide) and was interpreted visually.

Screening

All supernatants were initially screened against formalin-fixed tissue "sausages" which were assembled by juxtaposing previously diagnosed samples from individual patients into paraffin blocks [2]. Three of these sausages were formed from the palmar fascia of nine patients with Dupuytren's contracture and active stage disease [36]. A wide variety of normal adult and fetal tissue in three sausages and 21 individual paraffin blocks were screened. Once the selected clone was established, the supernantant was extensively screened against additional cases of Dupuytren's disease and myofibroblast-rich lesions. Slides for immunohistochemistry, subbed with chromatin, were prepared by using deparaffinized tissue sections cut 4 µm thick which did not require enzymatic predigestion. Pretreatment included 7 min in 3% hydrogen peroxide in methanol to elimate endogenous peroxidase activity and 20 min in 3% goat serum (Cederlane, Ontario, Canada) to quench nonspecific protein reactions. The avidin-biotin peroxidase method (Vector Laboratories, California) was followed by overnight incubation with the supernatnant diluted 1:2 with RPMI at room temperature [18]. Ten samples of Dupuytren's nodules were snap frozen and stored at −80°C. Prior to the avidin-biotin peroxidase technique, glass slides were coated with 0.05% polylysine to facilitate adherence of the 4 µm thick tissue sections. Pretreatment consisted of fixing the tissue in acetone and leaving it in 3% goat serum for 20 min to block nonspecific proteins.

Results and Discussion

The cells of a typical proliferative Dupuytren's nodule had elongated vesicular nuclei; irregularities of the nuclear membrane were barely visible at ×400 magnification. The cytoplasm was eosinophilic but microfilaments and cell connections were not seen (Fig. 1). By transmission electron microscope, the



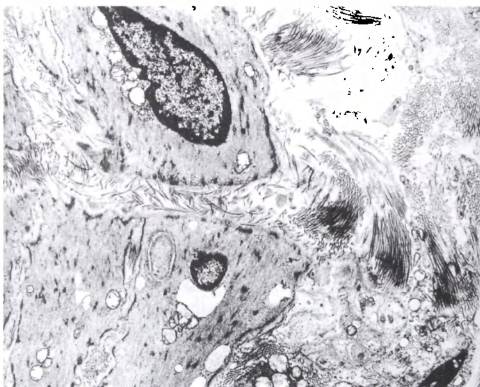


Fig. 1 *(above)*. Myofibroblasts in proliferative Dupuytren's nodule showing irregular nuclear membrane. Cytoplasmic filaments with densities are not discernible. Hematoxylin and eosin, ×400

Fig. 2 *(below)*. Transmission electron micrograph illustrating cells in proliferative Dupuytren's nodule. The cytoplasmic filaments with densities and cell to cell and cell to stromal connections are features typical of a myofibroblast

cytoplasmic microfilaments with densities and the cell to cell and cell to stroma connections were easily discernible. These ultrastructural features were typical of myofibroblasts (Fig. 2).

Using the 3C2G10.7 antibody, which was an IgM kappa immunoglobin, the cytoplasm of most of the cells in the Dupuytren's nodules was stained positively. The reaction was more intense with fresh, frozen unfixed tissue, but otherwise the pattern was identical to that observed in formalin-fixed, paraffin-embedded tissue, which revealed superior histologic and cytologic details.

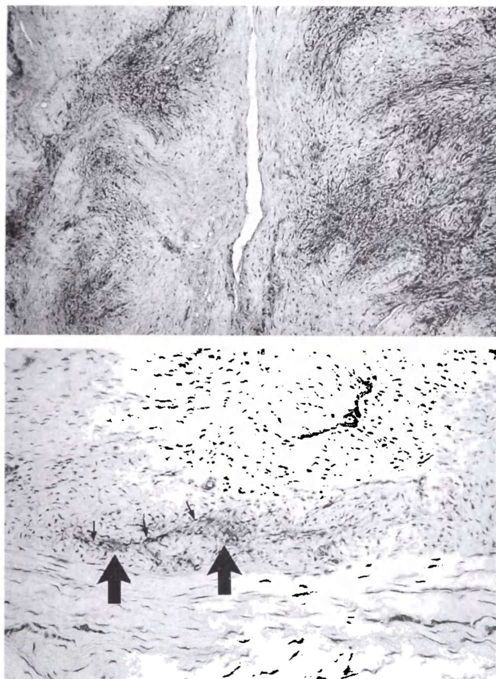


Fig. 3 *(above)*. Immunohistochemical staining of a proliferative Dupuytren's nodule, using 3C2G10.7 monoclonal antibody. Most cells in the nodule show positive cytoplasmic staining. Foci of positive cells are also noted peripheral to the main nodule (*right*). ×25

Fig. 4 *(below)*. A focus of positive cells (*large arrows*) peripheral to a Dupuytren's nodule, as demonstrated by immunohistochemical staining using 3C2G10.7 monoclonal antibody. Positive cells surround a capillary (*small arrows*). ×40

Microscopic foci of positively stained cells were observed peripheral to the main nodules in most of the Dupuytren's lesions (Fig. 3). In these areas, the cells had smaller nuclei and they appeared to be circumferential to capillaries that had a single layer of endothelial cells. (Fig. 4) The angiocentric arrangement of reactive cells was especially interesting in view of the fact that normal pericytes around capillaries were nonreactive to 3C2G10.7. These foci were inconspicuous under routine hematoxylin and eosin stain but we wondered if these were representative of incipient Dupuyren's nodules and propose that antigenic alteration of perivascular cells (possible pericytes or perivascular mesenchymal cells) could be an early detectable event with our antibody.

Fig. 5. Immunohistochemical staining of acute granulation tissue using 3C2G10.7 monoclonal antibody. Positive cells (*arrows*) are predominantly perivascular in location. ×40

Table 1. Immunohistochemical staining reaction with monoclonal antibody 3C2G10.7 on reactive and neoplastic lesions of myofibroblasts

Lesion	Results	Cases studied (*n*)
Dupuytren's disease	Staining of most cells in proliferative nodule; "satellite lesions" peripheral to the main nodule	30
Granulation tissue	Staining of pericapillary spindle cells in acute granulation tissue; positive cells found in fibrous base of chronic skin ulcer	10
Hypertrophic scar	Positive cells between collagen fibers	5
Colonic adenocarcinoma	Staining of occasional spindle-shaped stromal cells; positive cells surround neoplastic glands	2
Breast carcinoma	Staining of occasional spindle-shaped stromal cells	4
Desmoid	Staining of most tumor cells	2
Malignant fibrous histiocytoma	Staining of most tumor cells	4
Atypical fibroxanthoma	Staining of most tumor cells	2

Table 1 lists the results of immunohistochemical staining of additional pathologic conditions in which myofibroblasts have been reported as the principal cell component. In accordance with the original report of myofibroblasts in human granulation tissue [34], the 3C2G10.7 antibody revealed positively stained cells concentrated especially in the perivascular location in acute granulation tissue (Fig. 5). This was in contrast to the reaction with chronic skin ulcers and hypertrophic scars which demonstrated reactive cells oriented parallel to the skin surface throughout the fibrotic dermis (Fig. 6). The different location of myofibroblasts in acute granulation tissue and dermal scars concurred with suggestions of myofibroblasts being derived from

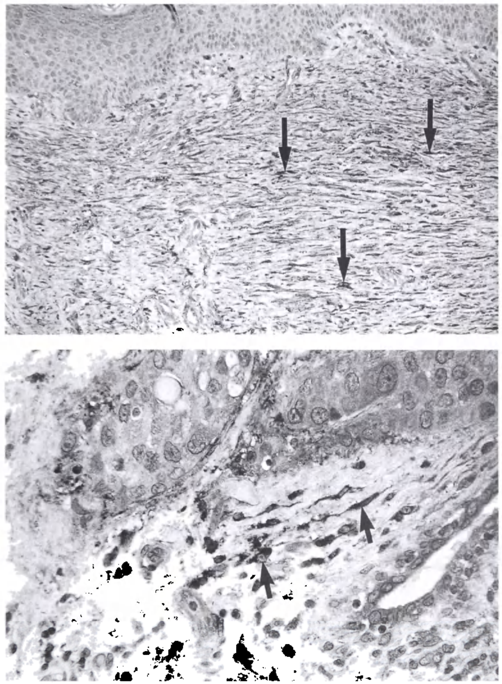


Fig. 6 *(above)*. Immunohistochemical staining of a dermal scar using 3C2G10.7 monoclonal antibody. Positive cells (*arrows*) are found throughout the fibrotic dermis and arranged parallel to the skin surface (epidermis on *top*). ×40

Fig. 7 *(below)*. Immunohistochemical staining of an invasive breast carcinoma using 3C2G10.7 monoclonal antibody. Positive spindle cells (*arrows*) are seen in the stroma surrounding nests of neoplastic cells. ×100

perivascular cells and providing fibrocontractive functions during wound healing [20]. It was significant to note that fibroblasts distant from the ulcer base were nonreactive. Examination of invasive breast and colonic adenocarcinomas showed reactive cells especially in the cellular desmoplastic stroma surrounding neoplastic glands (Fig. 7). Only a few positive cells were noted in the relatively acellular hyaline stroma of the breast carcinomas and there appeared to be staining of the collagen focally. In both types of malignancies, the results confirmed previous ultrastructural reports [10,27] and reflected the presence of myofibroblasts in the stroma of invading carcinomas. An abdominal desmoid tumor, atypical fibroxanthomas of the skin, and malignant fibrous histiocytomas were other fibroproliferative conditions that

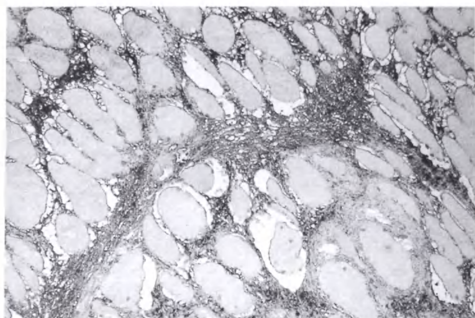


Fig. 8 *(above)*. Immunohistochemical staining of an abdominal desmoid tumor using 3C2G10.7 monoclonal antibody. Darkly stained positive tumor tissue is seen infiltrating nonstaining striated muscle fibers. ×25

Fig. 9 *(below)*. Immunohistochemical staining of a malignant fibrous histiocytoma using 3C2G10.7 monoclonal antibody. Many tumor cells show positive cytoplasmic staining. ×100

we screened for reactivity with the antibody. Most tumor cells, including morphologically atypical and giant cells in these lesions, were uniformly positive (Figs. 8, 9). In view of the prominence of myofibroblasts reported in previous ultrastructural studies [6,11,38,42] and the specific staining reaction of 3C2G10.7 on Dupuytren's nodules, granulation tissue, and dermal scars, a myofibroplastic lineage for these fibroproliferative tumors is strongly implicated.

On screening normal tissue with 3C2G10.7 (Table 2), reactive cells were noted in the tunica media of the aorta, medium size muscular artery, and coronary atherosclerotic plaque (Fig. 10). Such observations were again consistent with previous reports of the presence of myofibroblasts in these sites [13,15,16]. The remaining "control" sections tested negative with the exception of staining of

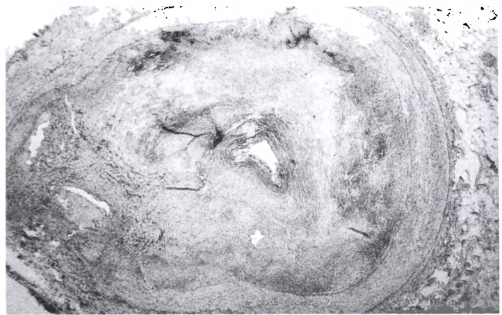


Fig. 10. Immunohistochemical staining of atherosclerotic coronary artery using 3C2G10.7 monoclonal antibody. Darkly stained positive cells are in the thickened tunica intima. There is marked luminal stenosis. ×12

Table 2. Immunohistochemical staining reaction with monoclonal antibody 3C2G10.7 on normal and fetal tissue

Tissue	Results
Myocardium	Negative
Lung	Negative
Liver	Negative
Aorta (atherosclerotic)	Positive cells in tunica media and atherosclerotic plaque
Coronary artery (atherosclerotic)	Positive cells in tunica media
Adrenal	Negative
Pancreas	Staining of islet cells
Striated muscle	Negative
Parotid	Negative
Testis	Negative
Skin and subcutaneum	Positive cells in eccrine sweat gland
Nerve	Negative
Thyroid	Negative
Spleen	Negative
Kidney	Negative
Fetal tissue (12–14 weeks)	Staining of chondrocytes and perichondral mesenchymal cells
Cartilage	Staining of chondrocytes
Bone	Negative
Plasma cells	Positive
Mast cells	Positive

mast cells, plasma cells, chondrocytes, pancreatic islet cells, eccrine sweat glands, and occasional epithelial cells. There was no explanation for this extraneous reactivity except to assume a nonspecific cross reaction that would not have untowards effects or influence on the practical use of 3C2G10.7 to identify myofibroblasts in tissue sections.

Myofibroblasts in Dupuytren's disease have been studied in the past with antibodies against vimentin, cytokeratin, desmin, actin isoforms [14,35,36,37,40], and connective tissue differentiation antigens [1]. The conclusions from these investigations were that myofibroblasts possess varied heterogenous cytoskeletal and actin compositions and that the antigenic constituent depends upon the basic pathologic process. Reactive myofibroblasts, for example, were found to be different from myofibroblasts of pathologic contractile conditions in their content of action isoforms [37]. Alternatively, the existence of phenotypic "subtypes" of myofibroblasts has been explained by theorizing that various nonmuscle connective tissue cells could modulate into a "myofibroblastic" state when contraction of local tissue was needed [13,20].

Our novel monoclonal antibody 3C2G10.7 has the capacity to react with myofibroblasts in a variety of reactive and neoplastic conditions, thereby defining an antigen common to cells showing myofibroblastic features and differentiation. The action of this antibody on Dupuytren's nodule revealed previously unrecognized early satellite lesions peripheral to the main nodule. The close proximity of these early lesions to vessels and studies on granulation tissue further suggested that early emerging myofibroblasts were located perivascularly. These results added credence to previous hypotheses that myofibroblasts were histogenetically associated with vascular smooth muscle cells [8,33,36]. Besides their unique ultrastructural features, we have concluded that myofibroblasts are antigenically distinct, and this cellular state of differentiation can be identified by the murine monoclonal antibody 3C2G10.7 at the light microscopic level using conventional immunohistochemical techniques.

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