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Angela Chiavegato · Marie-Luce Bochaton-Piallat
Emanuele D'Amore · Saverio Sartore · Giulio Gabbiani

Expression of myosin heavy chain isoforms in mammary epithelial cells and in myofibroblasts from different fibrotic settings during neoplasia

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Abstract The expression of smooth muscle (SM) and non-muscle (NM) myosin heavy chain (MyHC) isoforms has been studied in fibroblastic cells of different fibrotic lesions (hypertrophic scars, Dupuytren's nodules and stromal reaction to mammary carcinoma) and in epithelial cells of non-neoplastic and neoplastic mammary glands, using anti-myosin antibodies in immunofluorescence and Western blotting. Two antibodies were specific for SM-MyHC isoforms (SM1 and SM2) and three antibodies were directed against different sequences of NM-MyHC isoforms. Myofibroblasts containing SM-MyHC were present in a variable number of cases of the different lesions: 1 of 11 hypertrophic scars, 3 of 9 Dupuytren' s nodules and 20 of 25 breast cancers. The distribution of NM-MyHC sequences recognized by our antibodies was heterogeneous in fibroblasts from normal dermis and mammary stroma, but became homogeneous in myofibroblasts from all the pathological conditions examined. Moreover, the expression of these MyHC sequences differed in normal mammary epithelium when compared with invasive carcinoma. These results show that cellular modulation from fibroblast to myofibroblast may be accompanied by the appearance of SM-MyHC and is characterized by a uniform expression of MyHC of NM type, and that tumour progression in mammary epithelial cells may be paralleled by the disappearance of a specific NM-MyHC sequence. This suggests that MyHC modulation participates in the process of fibrosis as well as in the process of malignant epithelial transformation.

Key words Granulation tissue · Actin isoforms · Vimentin · Dupuytren's disease · Hypertrophic scar

A. Chiavegato · S. Sartore
Department of Biomedical Sciences, University of Padua, Via Trieste 75, I-35121 Padua, Italy

E. D'Amore
Institute of Anatomical Pathology, Via A. Gabelli 61, University of Padua, I-35121 Padua, Italy

S. Sartore
C.N.R. Unit for Muscle Biology and Physiopathology, Via Trieste 75, I-35121 Padua, Italy

G. Gabbiani (✉) · M.-L. Bochaton-Piallat
Department of Pathology, University of Geneva, CMU, 1, rue Michel-Servet, CH-1211 Geneva 4, Switzerland

Introduction

During the development of fibrotic lesions, including stromal reactions to epithelial tumours, fibroblastic cells modulate their phenotype and acquire smooth muscle (SM) cell features (for review see [37]). These modulated fibroblasts or myofibroblasts are probably responsible for retractile phenomena, common in fibrotic changes, as well as for the deposition of excessive amounts of extracellular matrix, the most deleterious consequence of these pathological settings [20, 40, 42]. The expression of contractile and cytoskeletal proteins has been useful in order to characterize the myofibroblastic phenotype [40]. It is accepted that the hallmark of myofibroblastic differentiation is the expression of α-SM actin [10, 14, 44], the actin isoform typical of SM cells and of vascular SM cells in particular [47]. Myofibroblasts can also express desmin [44], an intermediate filament protein which is a general muscle cell marker [31] and which appears in a relatively small proportion of cells during chronic fibrosis. Very little is known about the expression of myosin heavy chain (MyHC) isoforms in myofibroblasts, a point of interest since expression of SM-MyHC is recognized as a marker of terminal SM differentiation [13, 17, 25, 52]. During wound healing in both experimental animals and humans it has been shown that myofibroblasts do not express SM-MyHC [2, 14] and this has been used as an argument suggesting that myofibroblasts derive from fibroblasts [10, 14, 44]. However, it has recently been shown that SM-MyHC and other SM markers can be expressed by myofibroblastic cells under certain experimental conditions [6].

In the present study we have examined, by means of specific antibodies, the presence of SM-MyHC and

non-muscle (NM) MyHC in fibroblasts as well as in myofibroblasts during different clinical settings, including hypertrophic scars, fibromatosis and stromal reaction to mammary carcinoma. We have also studied the expression of NM-MyHC in non-neoplastic and neoplastic epithelial cells in the breast. Our results show that normal fibroblasts are characterized by a heterogeneous NM-myosin content and that myofibroblasts from fibrotic situations are heterogeneous as far as SM-MyHC expression is concerned. Moreover, mammary epithelial cells appear to show different NM-MyHC composition in non-invasive versus invasive situations; thus, SM- and NM-MyHC appear to be useful tools for the definition of the myofibroblastic phenotype. They may allow the definition of a hitherto unknown difference between malignant and non-malignant mammary epithelial cells.

Materials and methods

Tissue samples

Normal and pathological human tissues were obtained from surgical pathology material at the Department of Pathology, University of Geneva, Switzerland. Pathological specimens included: 11 hypertrophic scars, 9 Dupuytren's nodules, and 25 breast cancers [6 ductal carcinomas in situ (DCIS) and 19 invasive ductal carcinomas (IDC)]. All specimens were from different patients. Normal myometrium, skin, palmar aponeurosis and breast served as controls. Specimens were frozen in isopentane immersed in liquid nitrogen and stored at –80°C for immunofluorescence staining and biochemical analysis.

Antibodies

A panel of monoclonal and polyclonal antibodies which recognize different myosin isoforms was used (Table 1). This consisted of three monoclonal antibodies raised against human platelet myosin and recognizing NM-MyHC isoforms (NM-A9, NM-F6 and NM-G2) [3, 4]; a monoclonal antibody (SM-E7) [3]; and an affinity-purified rabbit polyclonal antibody (ABAM) [2] recognizing both SM1 and SM2. In addition, three monoclonal antibodies were used for double immunofluorescence staining with the anti-myosin antibodies: anti-αSM-1 specific for α-SM actin [43], anti-vimentin (Dako, Glostrup, Denmark) and anti-cytokeratin recognizing all cytokeratins (kindly provided by Prof. W.W. Franke, Heidelberg, Germany).

Purification of NM-actomyosin, proteolysis and peptide mapping

NM-actomyosin was purified from human platelets according to Pollard et al. [33]. All extraction steps were carried out in the presence of protease inhibitors: 100 μg/ml pepstatin A, 20 μg/ml leupeptin, 0.5 μg/ml soybean trypsin inhibitor and 0.1 mM phenylmethylsulphonyl fluoride (PMSF).

Production of major fragments of NM-actomyosin was performed by enzymatic digestion according to Whalen et al. [51] with minor modifications [9]. NM-actomyosin was digested with α-chymotrypsin (4:1, vol:vol; Worthington, Freehold, N.J., USA) in the presence of 0.1% sodium dodecyl sulphate (SDS). Digestion was blocked by adding 1 mM PMSF and 1% SDS and boiling the samples for 2 min.

Immunofluorescence staining

Immunofluorescence staining was performed on 4-μm-thick cryostat sections applied to silan (3-aminopropyl-triethoxysilan; Fluka, Buchs, Switzerland) pre-coated glass slides. Tissue sections were air-dried at room temperature for 2 h and then incubated at room temperature for 1 h with one or two of the primary antibodies described above. After rinsing in phosphate-buffered saline, the sections were incubated at room temperature for 1 h with rabbit anti-mouse or goat anti-rabbit IgG conjugated with tetramethylrhodamine isothiocyanate (Jackson ImmunoResearch, Pa., USA) or dichlorotriazynil-amino-fluorescein (Jackson ImmunoResearch). To minimize cross-reactions, secondary antibodies were passed on a solid immunoabsorbent of human serum cross-linked with glutaraldehyde. Controls were performed using non-immune rabbit or mouse IgG instead of the primary antibody. Slides were mounted in buffered polyvinyl alcohol [28].

Photographs were taken with a Zeiss Axiophot fluorescence photomicroscope (Zeiss, Oberkochen, Germany) equipped with epi-illumination and specific filters for fluorescein and rhodamine on Kodak T-max 400 black and white films (Eastman Kodak, Rochester, N.Y., USA).

SDS-PAGE and Western blotting

Sections 20 μm thick were cut from frozen specimens on the basis of immunofluorescence results on preceding sections; they were dissolved in sample buffer containing 1% SDS, 1% dithiothreitol, 1 mM PMSF, 1 mM *Nα-P*-tosyl-L-arginine methylester in 0.4 M TRIS-HCl, pH 6.8 [26]. After sonication, samples were boiled for 3 min and centrifuged at 12,000 *g* for 10 min; the supernatants were stored at –80°C. Protein concentration was measured according to Bradford [5].

In order to separate the different myosin isoforms, proteins were electrophoresed on a 5% SDS-PAGE containing 30% acrylamide and 0.4% bisacrylamide [21]. Proteolytic fragments were separated on a 12.5% SDS-PAGE. Gels were stained with Coomassie blue.

Table 1 Antibodies used in our experiments (*mAb* monoclonal antibody, *pAb* polyclonal antibody, *SM* smooth muscle, *NM* non-muscle, *MyHC* myosin heavy chain, *ABAM* affinity-purified rabbit polyclonal antibody)

Antibody	Antigen	Reactivity	References
Anti-αSM-1 (mAb)	*N-terminalpeptide of α-SMactin*	α-SM actin	[43]
ABAM (pAb)	*Bovine aorta actomyosin*	SM-MyHC1 and 2	[2]
SM-E7 (mAb)	*Bovine aorta actomyosin*	SM-MyHC1 and 2	[3]
NM-A9 (mAb)	*Human platelet actomyosin*	NM-MyHC, 196 KD	[4]
NM-F6 (mAb)	*Human platelet actomyosin*	NM-MyHC, 196 KD	[4]
NM-G2 (mAb)	*Human platelet actomyosin*	Two NM-MyHC isoforms around 196 KD[a]	[4]

[a] In human tissues (see [17])

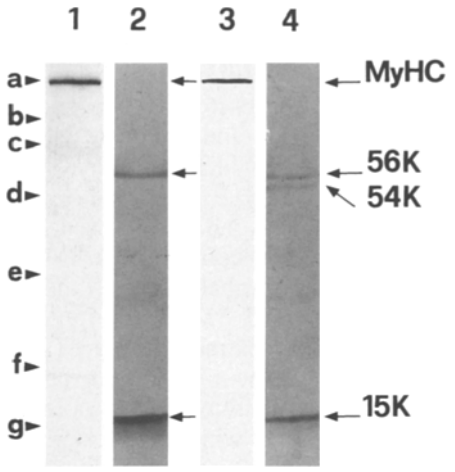

Fig. 1 Western blotting analysis with non-muscle (NM)-G2 (*lanes 1, 2*) and NM-A9 (*lanes 3, 4*) of undigested human platelet NM-actomyosin (*lanes 1, 3*) and chymotryptic digested NM-actomyosin (lane 2, 4) after 12.5% SDS-PAGE. *Arrowheads* on the *left* mark the molecular weight standards corresponding to: *a* myosin heavy chains (MyHC, 200 kDa); *b* phosphorilase B (92.5 kDa); *c* bovin serum albumin (66.2 kDa); *d* ovalbumin (45 kDa); *e* carbonic anhydrase (31 kDa); *f* soybean trypsin inhibitor (21.5 kDa); and *g* lysozime (14.4 kDa). NM-G2 recognizes two fragments of 56 and 15 kDa, while NM-A9 reacts with three fragments of 56, 54 and 15 kDa (*arrows*)

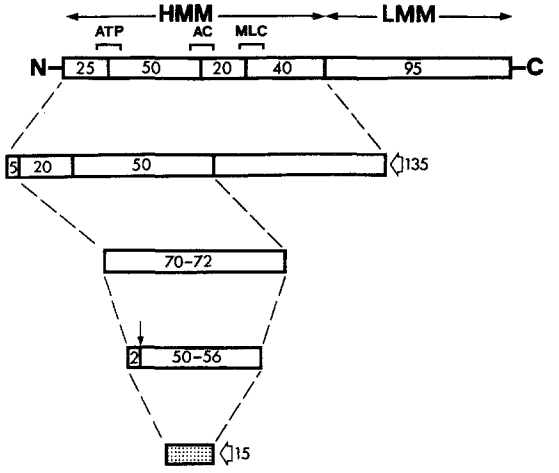

Fig. 2 Schematic representation of myosin antigenic epitopes localization recognized by NM-A9, NM-F6 and NM-G2. Numbers represent molecular weights of fragments; *small arrow* in the 50–56 kDa fragment indicates the possible cleavage site which gives rise to the 54 kDa peptide recognized by NM-A9 antibody. (*HMM* heavy meromyosin, *LMM* light meromyosin, *ATP* ATP-binding site, *AC* actin-binding site, *MLC* myosin light-chain binding site)

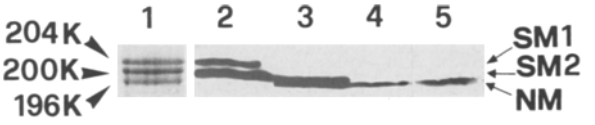

Fig. 3 Western blotting analysis with smooth muscle (SM)-E7 (*lane 2*), NM-G2 (*lane 3*), NM-F6 (*lane 4*) and NM-A9 (*lane 5*) after 5% SDS-PAGE of crude myosin extracted from human myometrium (*lane 1*). SM-E7 recognizes the two SM-MyHC at 200 and 204 kDa (*lane 2*). The three NM-MyHC antibodies react with NM-MyHC of 196 kDa (*lanes 3–5*). Note that NM-G2 appeared as a doublet of two close bands (*lane 3*). (*SM1* SM-MyHC of 204 kDa; *SM2* SM-MyHC of 200 kDa, *NM* NM-MyHC of 196 kDa)

Western blotting was performed according to Towbin et al. [46] with minor modifications [3]. Proteins separated on SDS-PAGE were transferred to 0.45 μm nitrocellulose paper (Schleicher & Schuell, Dassel, Germany) which was incubated with the different myosin antibodies. A second incubation with horseradish-peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (Nordic Immunological Laboratories, Tilburg, Netherlands) was performed, and enhanced chemiluminescence (Amersham, Buckinghamshire, UK) was used for detection.

Results

Biochemical characterization of anti-NM-myosin antibodies and myosin isoform; composition of normal and pathological tissues

In order to define the epitopes of our monoclonal NM-MyHC antibodies better, fragments of human platelet actomyosin were analysed by means of Western blotting. Figure 1 shows that both NM-A9 and NM-G2 recognized a single band whose relative migration was around 200 kDa (see Fig. 3 for details); after chymotryptic digestion, NM-A9 recognized three fragments of 56, 54 and 15 kDa; NM-G2 only recognized the 56 and 15 kDa fragments. NM-F6 showed a pattern of reactivity similar to that of NM-G2 (data not shown). Proteolytic cleavage performed at a low chymotryptic-protein ratio showed the presence of reactive 135 and 70–72 kDa peptides (Capriani et al., to be published), probably corresponding to the head portion of the MyHC [41]. Figure 2 shows a schematic representation of our interpretation of epitope localization constructed from the results of our digestion experiment. It is known that α-chymotrypsin cuts heavy meromyosin into a fragment of 64 kDa at the C-terminal end and another fragment of 70–72 kDa at the N-terminal end; this last fragment undergoes a further cleavage, producing a 50–56 kDa fragment, as previously demonstrated using turkey gizzard [34, 41] and human brain myosins [23]. The epitopes of all our antibodies are localized in this 50–56 kDa fragment. The 15 kDa and the 2 kDa fragments derive from the digestion of the 50–56 kDa fragment. The NM-A9 epitope appears to be localized in the portion of the 15 kDa fragment not including the additional 2 kDa fragment, while NM-F6 and NM-G2 recognize epitopes in the 2 kDa fragment.

Electrophoresis of extracts from normal human myometrium on a 5% SDS-PAGE allowed the resolution of at least three MyHC bands (Fig. 3, lane 1): one of 204 kDa corresponding to SM1, a second of 200 kDa containing SM2 [17] and one or two B-type NM-MyHC isoforms [30], and a third of 196 kDa containing at least

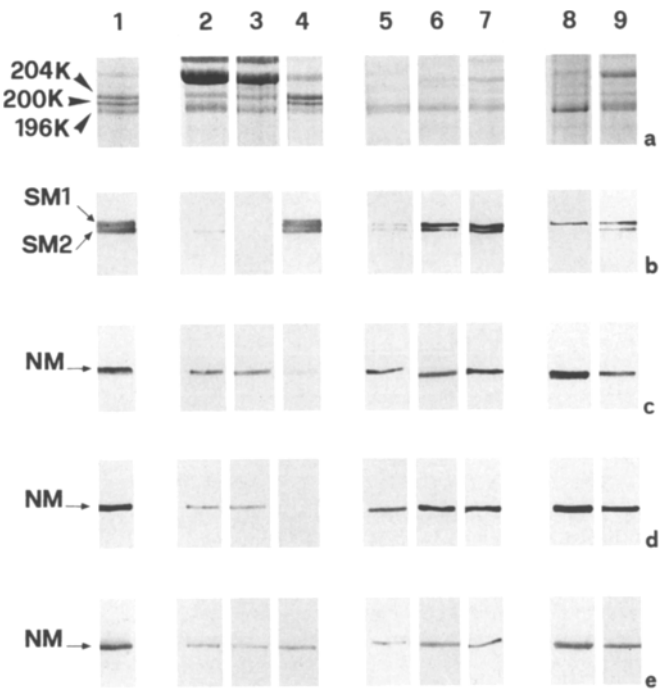

Fig. 4a–e Western blotting analysis with affinity-purified rabbit polyclonal antibody (ABAM; **b**), NM-A9 (**c**), NM-F6 (**d**) and NM-G2 (**e**) after 5% SDS PAGE (**a**) of human myometrium (*lane 1*), normal human skin (*lane 2*), hypertrophic scars (*lanes 3, 4*), normal palmar aponeurosis (*lane 5*), Dupuytren's nodules (*lanes 6, 7*), normal breast (*lane 8*) and invasive ductal (IDC) breast cancer (*lane 9*). All specimens express the two SM-MyHC except for one case of hypertrophic scar, which is negative for ABAM (**b**, *lane 3*), and the normal breast, which contains only SM1 (**b**, *lane 8*). The three NM-MyHC antibodies react with all normal and pathological tissues. (*SM1* SM-MyHC of 204 kDa, *SM2* SM-MyHC of 200 kDa, *NM* NM-MyHC of 196 kDa)

two different A-type NM-MyHC isoforms [23]. ABAM and SM-E7 antibodies recognized the two heavier bands containing SM-MyHC isoforms (Fig. 3, lane 2); NM-A9 and NM-F6 antibodies reacted with a single band of 196 kDa (Fig. 3, lanes 4 and 5), while NM-G2 recognized a band of 196 kDa, which occasionally appeared as a doublet (Fig. 3, lane 3).

Western blotting with ABAM showed that extracts from normal human skin contain both SM-MyHC isoforms (Fig. 4b, lane 2) probably due to the presence of SM cells from arrector pili SM in addition to small vessels. Hypertrophic scar extracts appeared negative for ABAM (Fig. 4b, lane 3) with a single exception, which was strongly positive in immunofluorescence with our SM-MyHC antibodies and which by Western blotting expressed both SM1 and SM2 isoforms (Fig. 4b, lane 4). In this last case, two very close bands were detected at 204 kDa, while only one band was present in normal human skin; these might correspond to the two SM1 isoforms observed in different SM tissues [1, 22]. Normal palmar aponeurosis and Dupuytren's nodules showed both SM-MyHC isoforms (Fig. 4b, lanes 5–7) which may be explained in part by the great number of small blood vessels and also by nodular myofibroblasts (see immunoflorescence results). Normal breast only expressed the SM1 isoform (Fig. 4b, lane 8) probably due to the presence of myoepithelial cells. IDC extracts showed the presence of both SM-MyHC (Fig. 4b, lane 9), which is probably due to stromal myofibroblasts of the desmoplastic reaction, since IDC virtually contain no myoepithelial cells.

Western blotting of normal and pathological tissues showed that only the sample of hypertrophic scar containing SM myosin was weakly reactive with NM-A9 and practically negative with NM-F6 (Fig. 4c, d, lane 4). In all the other samples NM-A9, NM-F6 and NM-G2 re-

Table 2 Immunoreactivity of normal and pathological human tissues with anti-cytoskeletal protein antibodies as determined by indirect immunofluorescence (– negative staining, + positive staining, +/– rare positive cells)

Human tissues	Anti-αSM-1	ABAM	NM-A9	NM-F6	NM-G2
Normal skin					
Epithelium	–	–	+	+	+
Dermis	–	–	+	+	±
Arrector pili muscles	+	+	+	±	+
Normal breast					
Glandular epithelium	–	–	+	+	+
Myoepithelium	+	+	+	+	+
Stroma	–	–	+	+	±
Hypertrophic scars					
Stroma	+	–[a]	+	+	+
Dupuytren's nodules					
Stroma	+	+[a]	+	+	+
DCIS					
Neoplastic cells	–	–	+	+	+
Stroma	+	±	+	+	+
IDC					
Neoplastic cells	–	–	+	±	–[a]
Stroma	+	+	+	+	+

[a] See details in Results

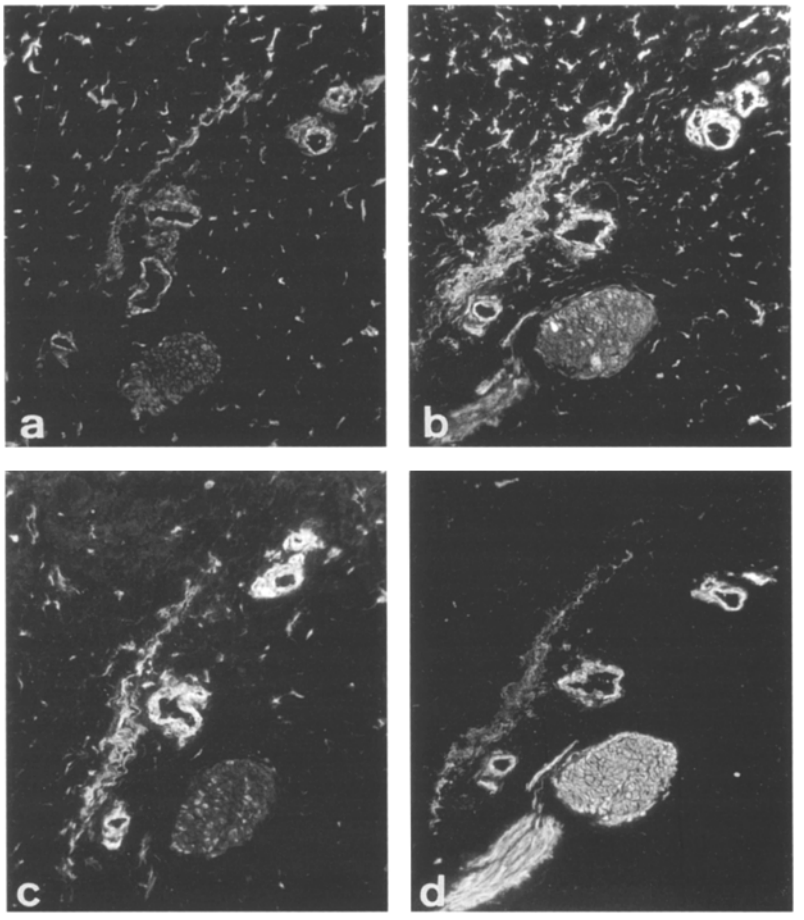

Fig. 5 Immunofluorescence stainings of human normal skin dermis with anti-vimentin (**a**), NM-A9 (**b**), NM-F6 (**c**), and NM-G2 (**d**). While NM-A9 stains the majority of dermal fibroblasts, NM-F6 stains only a few and NM-G2 does not stain these cells. The arrector pili muscles are differently labelled by the three NM-MyHC antibodies. × 640

acted with the 196 kDa band (Fig. 4c–e). In breast cancer, NM-G2 frequently appeared as a doublet of very close bands.

Cellular distribution of myosin isoforms and other cytoskeletal proteins

Normal human skin and mammary gland

Anti-vimentin used on normal human dermis showed a general staining of mesenchymal cells, SM cells of small blood vessels and arrector pili SM (Fig. 5a). The three NM-MyHC antibodies strongly reacted with small vessels and epithelium but exhibited heterogeneous staining in fibroblasts and arrector pili SM cells (Figs. 5b–d, 6b–d). All mesenchymal cells were labelled with NM-A9 (Figs. 5b, 6b); about 30% of them were positive for NM-F6 (Figs. 5c, 6c); and they were practically negative for NM-G2 except for rare cells in the papillary dermis (Figs. 5d, 6d). The staining pattern of arrector pili cells was irregular with NM-A9, dotted with NM-F6 and uniform with NM-G2 (Fig. 5b–d). The results of immunofluorescence staining with ABAM, NM-A9, NM-F6, NM-G2 and anti-αSM-1, on normal and pathological tissues, are summarized in Table 2.

In the mammary gland, the three NM-MyHC antibodies reacted strongly with epithelium, myoepithelium and vessels and they stained stromal fibroblasts in a manner similar to what was observed in the dermis. NM-A9 stained all fibroblasts (Fig. 7b); NM-F6 stained a proportion of them (Fig. 7c). NM-G2 only reacted with fibroblasts around acini (Fig. 7d).

In all specimens studied anti-αSM-1, SM-E7 and ABAM stained small blood vessels, arrector pili SM and myoepithelium (Figs. 6a, 7a).

Hypertrophic scars, Dupuytren's nodules and stromal reaction to mammary carcinoma

All cases of hypertrophic scar were characterized by the presence of nodular structures localized in the deep dermal layer containing nests of spindle-shaped fibroblasts and surrounded by abundant collagen [15]. Dupuytren's nodules consisted of collagen bands with numerous interwoven fibroblastic cells [39]. These fibrotic populations displayed a uniform immunoreactivity to NM-A9, NM-F6 and NM-G2 (Fig. 6f–h, j–l) and a variable proportion of cells was stained by anti-αSM-1 (data not shown). Myofibroblasts of hypertrophic scar were negative for ABAM and SM-E7 (Fig. 6e) with the exception of one case, which showed diffuse and strong staining

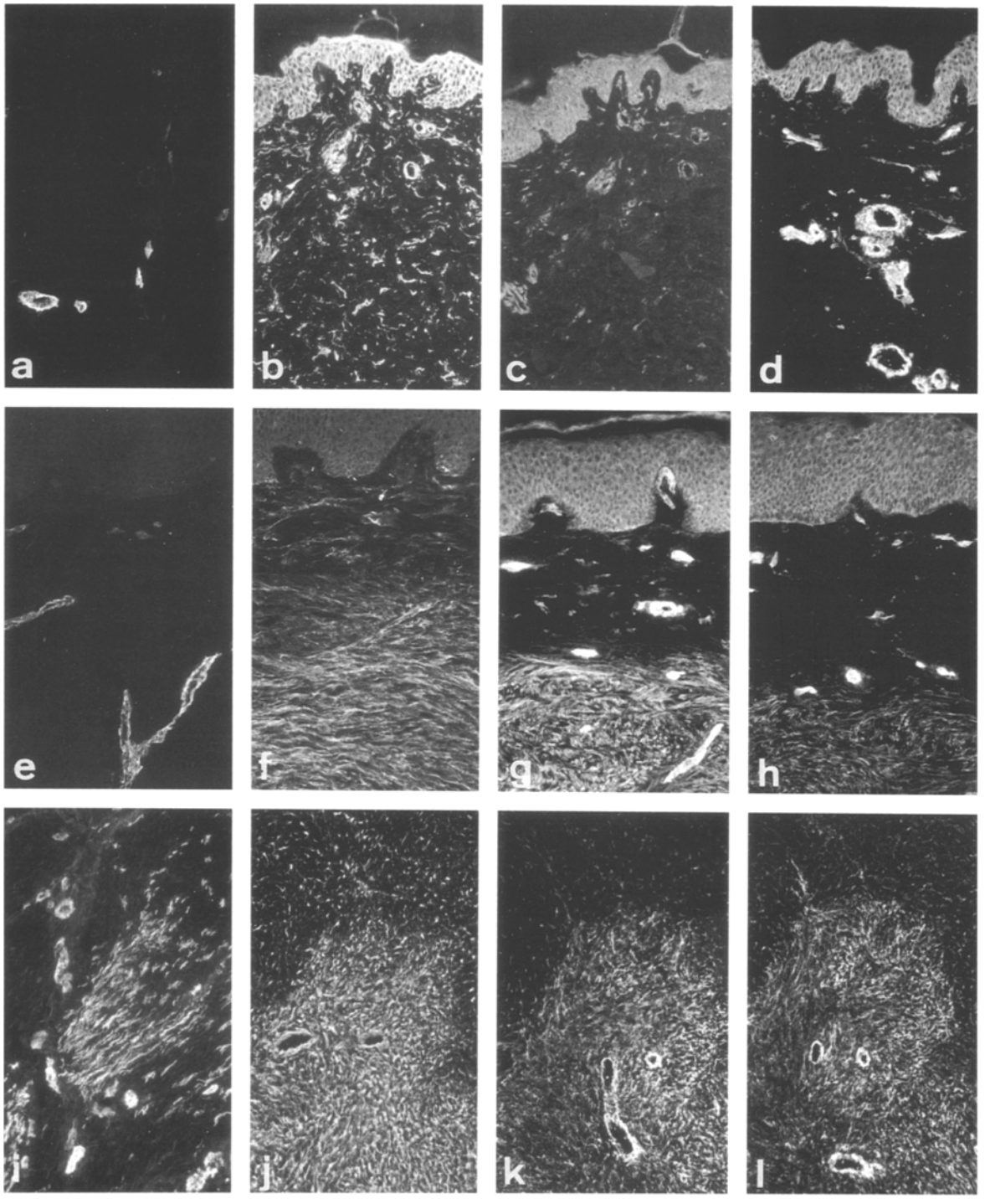

Fig. 6 Immunofluorescence stainings of normal human skin (**a–d**), hypertrophic scars (**e–h**) and Dupuytren's nodules (**i–l**) with ABAM (**a, e, i**), NM-A9 (**b, f, j**), NM-F6 (**c, g, k**) and NM-G2 (**d, h, l**). Cells of the fibrotic lesions are strongly stained with NM-A9 (**f, j**) similar to dermis fibroblasts (**b**) but also with NM-F6 (**g, k**) and NM-G2 (**h, l**). A Dupuytren's nodule expresses SM-MyHC (**i**). ×320

with both antibodies (data not shown). In 3 out of 9 Dupuytren's cases, myofibroblasts were focally positive for ABAM and SM-E7 (Fig. 6i).

Breast cancers were divided histologically in two main groups: DCIS and IDC. The DCIS specimens were characterized by ducts filled with neoplastic cells surrounded by a thin layer of myoepithelial cells. The stroma of these tumours had a trabecular appearance and exhibited elongated fibroblastic cells [42]. A marked inflammatory infiltration was rarely seen. Immunofluorescence staining with anti-cytokeratins, anti-αSM-1 and

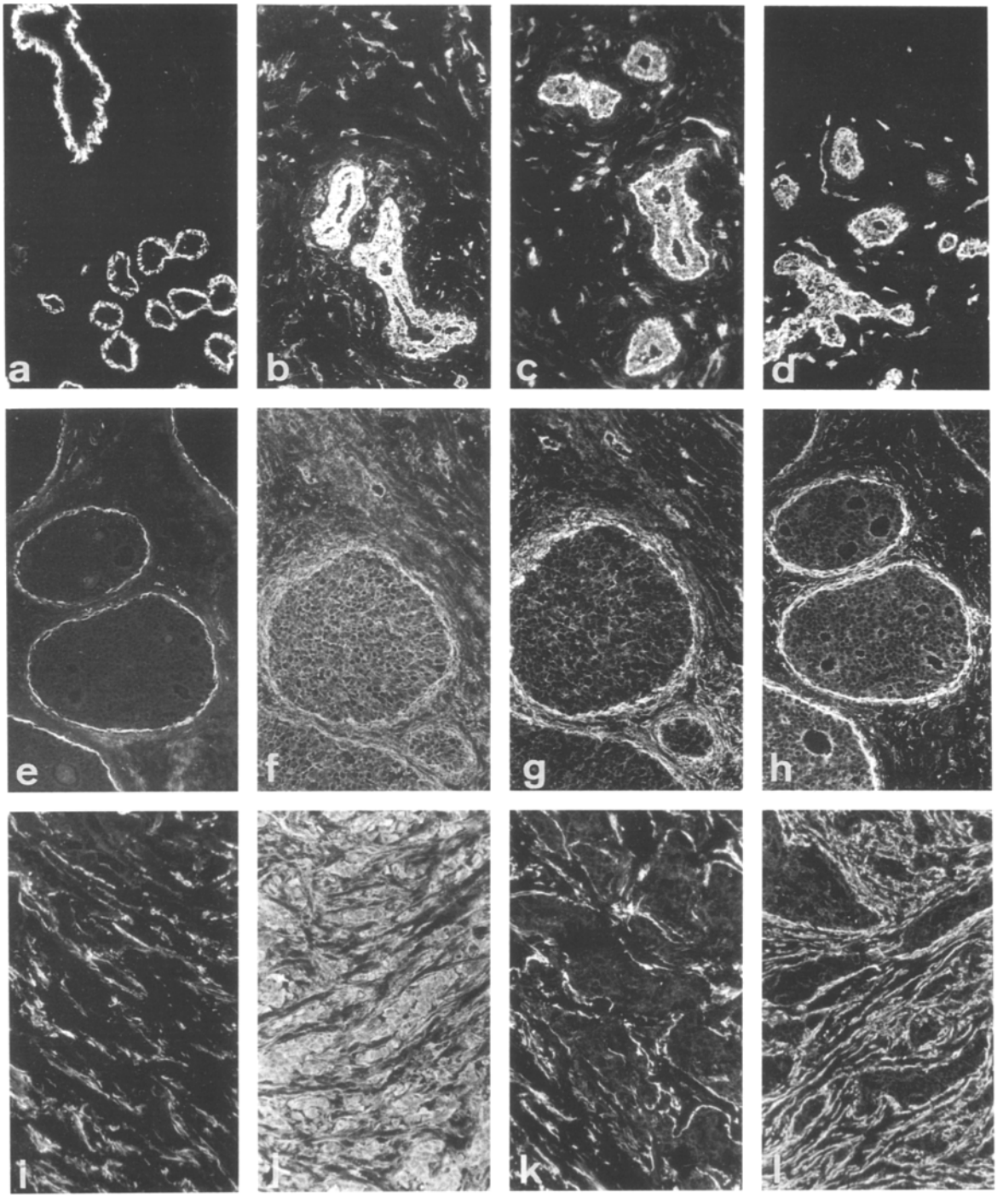

Fig. 7 Immunoflurescence stainings of human normal breast (**a–d**), ductal carcinoma in situ (DCIS; **e–h**) and IDC (**i–l**) breast cancer with ABAM (**a, e, i**), NM-A9 (**b, f, j**), NM-F6 (**c, g, k**) and NM-G2 (**d, h, l**). Fibroblasts of stromal reactions react with the three NM-MyHC antibodies (**f–h, j–l**). IDC stromal cells display a diffuse reactivity with ABAM while only a few stromal cells in DCIS are positive for this antibody. Note that NM-F6 and NM-G2 staining is weak in the neoplastic cells of IDC (**k, l**). ×320

ABAM identified myoepithelial cells. Stromal cells were always stained by the three anti-NM-myosin antibodies (Fig. 7f–h). Several stromal fibroblasts were positive for anti-αSM-1, while only a few were also positive for ABAM and SM-E7 (Fig. 7e). These positive cells were preferentially localized in the vicinity of neoplastic ducts. In the two comedo DCIS, SM-MyHC-positive myofibroblastic cells were not present but fibroblasts, strongly positive for NM-G2, were visible around neoplastic ducts.

The 19 IDC showed a considerable histological variability; they were chosen irrespective of their histologi-

cal grade but were all characterized by a clear desmoplastic reaction. In all IDC a high proportion of stromal cells were positive for anti-αSM-1; 16 of 19 cases showed staining for ABAM and SM-E7 (Fig. 7i); in 5 cases this staining was limited to a low proportion of stromal cells. Stromal cells were always stained by all anti-NM-MyHC antibodies (Fig. 7j–l).

We also examined the distribution of NM-MyHC in epithelial cells in normal and neoplastic mammary glands. Double immunofluorescence staining of normal mammary gland with anti-NM-MyHC and anti-cytokeratin antibodies showed that epithelial cells were always positive for NM-A9, NM-F6 and NM-G2 (Fig. 7b–d). Epithelial cells in DCIS were weakly stained with NM-F6 and NM-G2 compared to normal epithelium (Fig. 7 g, h). Staining for NM-F6 and NM-G2 became minimal (5 cases) or negative (7 cases) in IDC (Fig. 7k, l). NM-A9 was always uniformly positive in DCIS and IDC neoplastic cells (Fig. 7f, j).

Discussion

Temporary differentiation of myofibroblasts represents a useful event during healing by second intention [40]. When healing is completed myofibroblasts disappear, allegedly through apoptosis, and scar formation ensues [8, 10]. In other more permanent pathological situations, including hypertrophic scar, fibromatosis, organ fibrosis and stromal reaction to epithelial tumours, myofibroblast persistence appears to represent a damaging event, although the role of the stromal reaction in controlling tumour growth is still controversial [16, 29, 48]. In these conditions myofibroblasts have been shown to express SM markers more importantly, both in number and quantity, than in the morphologically similar myofibroblasts developing during wound healing [37, 38, 40, 44]. Our results confirm and extend these previous studies, bringing new data on MyHC expression in myofibroblastic cells in different pathological settings.

It has been reported that SM-MyHC are not expressed in morphologically typical myofibroblasts present during experimental wound healing [2, 14]. Our results show that, with a single exception, hypertrophic scars also do not express SM-MyHC whereas these molecules appear to be expressed in myofibroblasts of Dupuytren's nodules and become a feature of myofibroblasts in practically all cases of stromal reaction to mammary carcinoma. This further supports the notion that myofibroblasts (and normal fibroblasts) are heterogeneous in their biochemical features and that they may acquire phenotypic features similar to those of SM cells, including the expression of SM-MyHC, under appropriate stimulation [24, 27, 37]. This feature is not only true of stromal reaction to epithelial tumours. Myofibroblasts expressing SM-MyHC have been described in human scars following myocardial infarction [49] and in the submesothelial region of hypertrophied rabbit bladder after mechanical obstruction [6]. Several questions still remain open, such as which factors regulate SM-MyHC expression in different myofibroblasts. Previous work has shown that transforming growth factor β1, heparin and granulocyte-macrophage colony stimulating factor tend to stimulate the expression of α-SM actin in fibroblastic cells, whereas γ-interferon tends to decrease it [11, 12, 32, 35, 36, 50]. Other well-accepted mediators of inflammatory changes such as tumour necrosis factor or interleukin-1 do not seem to affect this variable [36]. All these results show that, irrespective of the mechanism, fibroblastic cells can undergo a phenotypic modulation which results in the acquisition of SM cell differentiation markers. Another puzzling question is whether myofibroblasts expressing SM-MyHC should be still considered to be fibroblastic cells or whether they have reached complete SM differentiation. SM-MyHC are assumed to be markers of terminal SM differentiation. Although other markers such as caldesmon should be tested in order to define precisely the degree of differentiation of stromal fibroblastic cells, our results indicate that in some cases these stromal cells, which under normal conditions do not express SM cell markers, can differentiate into SM cells. This process corresponds to the definition of metaplasia.

Our results also furnish more data on the expression of NM-MyHC in fibroblastic cells and in mammary epithelial cells under normal conditions and in different pathological states. Our monoclonal antibodies have been produced by sensitizing mice with human platelet myosin. They recognize NM-MyHC but the exact epitopes remain unknown. The experiments of partial digestion furnish new information on their nature and suggest that the epitopes recognized by NM-A9, NM-F6 and NM-G2 are localized at one of the two extremities of the 56 kDa NM myosin fragment, which derives in turn from the 70–72 kDa fragment. It is conceivable that they are localized in a region close to the actin-binding site or to the adenosine triphosphate-binding site (see Fig. 2), which has been shown to be structurally distinct in different NM-MyHC isoforms [45]. Further information about the targets of NM-A9, NM-F6 and NM-G2 have been obtained from immunofluorescence analysis. Previous studies performed in rabbit have shown that these antibodies have a tissue-specific distribution and stain different cells during aorta [19] or bladder [7] development, as well as in various pathological conditions [7, 18, 53]. Moreover, the three antibodies exhibited different patterns of intracellular distribution in cultured rabbit SM cells [19], bovine endothelial cells [4] and human dermal fibroblasts (unpublished data).

Though we cannot exclude the possibility that the three different monoclonal anti-NM-MyHC antibodies recognize antigenic epitopes on the same molecule, there are several observations compatible with the possibility that they identify distinct NM myosin isoforms. Firstly, in the aorta of adult animals NM-G2 stains all medial SM cells, while NM-F6 is down-regulated during development [17]; secondly, in normal human dermis and in mammary stroma the anti-NM myosin antibodies show

different patterns of reactivity in fibroblastic cells and arrector pili SM cells (see Results); and finally, in IDC neoplastic cells NM-G2 and NM-F6 become virtually negative, whereas NM-A9 remains strongly positive (see Results). Selective masking of antigenic epitopes cannot account for the differences observed in these immunostaining patterns, since pre-treatment of cryosections with denaturants does not change the immunoreactivity with the three antibodies.

Whatever the interpretation of the specificity of our NM-MyHC antibodies, our results show that each has a distinct distribution in normal human dermis and arrector pili SM and that this distribution changes in fibroblastic cells during pathological situations. Thus, in normal tissues NM-A9 significantly stains fibroblasts, whereas in fibrotic conditions, including stromal reaction to mammary carcinoma, the MyHC recognized by NM-G2 becomes the most highly expressed. Finally, while all NM-MyHC antibodies recognize normal mammary epithelium with a similar pattern, NM-F6 and NM-G2 react very weakly with neoplastic cells, particularly of IDC.

In conclusion, our data extend the notion of fibroblast and myofibroblast heterogeneity and show that MyHC expression is an additional marker of fibroblast adaptation to pathological stimuli. SM-MyHC can be expressed by myofibroblastic cells, particularly during stromal reaction to epithelial tumours. The change of MyHC isoform expression is not limited to fibroblastic cells but involves epithelial cells, being particularly evident in invasive neoplastic as compared with normal mammary epithelial cells. The use of MyHC antibodies appears to represent a useful adjunct to the tools defining the phenotypic features of mesenchymal and epithelial cells.

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