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Differential Tissular Expression and Localization of Type IV Collagen α1(IV), α2(IV), α5(IV), and α6(IV) Chains and Their mRNA in Normal Breast and in Benign and Malignant Breast Tumors

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SUMMARY: Type IV collagen, the major component of basement membrane (BM), is composed of six genetically distinct α chains. We investigated the cellular regulation and origin of these α(IV) chains in normal and neoplastic breast tissues by immunohistochemistry by using α(IV) chain-specific antibodies and by in situ hybridization. In normal breast, α1(IV) and α2(IV) chains were stained in all BM, whereas α5(IV) and α6(IV) chains were restrictively localized in a linear pattern in the BM of the mammary gland. Similar immunostaining profiles were observed in benign breast tumors and in the intraductal components of invasive ductal carcinoma. However, in invasive ductal carcinoma, α1(IV) and α2(IV) chains were discontinuously or negatively stained in the cancer cell nests, and the assembly of α5(IV) and α6(IV) chains into the BM was completely inhibited. Coexpression of α5(IV) and α6(IV) chains was related to the localization of α-smooth muscle actin (α-SMA)–positive myoepithelial cells. By in situ hybridization, in fibroadenoma and invasive ductal carcinoma, the signals for α1(IV) and α2(IV) mRNA were abundant in stromal cells. However, the signals for α5(IV) and α6(IV) mRNA were not seen in any of these cells. In contrast, in intraductal papilloma, coexpression of α1(IV)/α2(IV) mRNA and α5(IV)/α6(IV) mRNA was identified in epithelial cells. The results indicate that the mammary gland forms a second network of BM composed of α5(IV)/α6(IV) chains, in addition to the classic network of α1(IV)/α2(IV) chains. The expression of type IV collagen α chains seems to be differentially regulated by the epithelial-myoepithelial interaction and to be associated with the invasive potential of breast cancer. (*Lab Invest 1999, 79:281–292*).

Basement membrane (BM), a continuous sheet of extracellular matrix, separates epithelial cells from the underlying stroma. BM plays important roles both in biological functions such as cell adhesion, cell differentiation, tissue repair, and in pathologic events such as cancer cell invasion and metastasis (Liotta and Stetler-Stevenson, 1991; Rohrbach and Timpl, 1993). Type IV collagen is one of the major structural components of BM. Type IV collagen molecule primarily consists of two α1(IV) chains and one α2(IV) chain. The heterotrimer, $[\alpha1(IV)]_2$ α2(IV), forms a macromolecular network in which the molecules are crosslinked by means of their C- and N-terminal ends (Timpl, 1989). Recently, other subunits of type IV collagen, α3(IV) - α6(IV) chains, have been characterized by protein chemistry (Butkowski et al, 1990; Wieslander et al, 1985) and molecular cloning (Leinonen et al, 1994; Mariyama et al, 1994; Oohashi et al, 1994; Soininen et al, 1988; Zhou et al, 1992). The genes encoding the six distinct α chains are paired by two on three different chromosomes in a head-to-head arrangement: the α1(IV) gene (COL4A1) and the α2(IV) gene (COL4A2) are localized on chromosome 13q34 (Soininen et al, 1988); the α3(IV) gene (COL4A3) and the α4(IV) gene (COL4A4) are on chromosome 2q36 (Mariyama et al, 1994); and the α5(IV) gene (COL4A5) and the α6(IV) gene (COL4A6) are on chromosome Xq22 (Oohashi et al, 1994; Zhou et al, 1993). Immunohistochemical studies have shown that α3(IV) - α6(IV) chains are expressed in a tissue-specific manner in human tissues. α3(IV), α4(IV), and α5(IV) chains were detected in the glomerular BM, alveolar BM, and some parts of the tubular BM in the kidney. α5(IV) and α6(IV) chains were localized in the BM of the epidermis, smooth muscle cells, prostate glands and developing intestine (Dehan et al, 1997; Mariyama et al, 1994; Ninomiya et al, 1995; Sado et al, 1995; Simoneau et al, 1998; Tanaka et al, 1997).

In normal mammary gland, the ductal-lobular epithelial system is composed of two types of epithelial lining cells, ie, the inner epithelium and the outer

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Table 1. Results of Immunohistochemical Studies*

Breast disease	Number of cases	α1(IV)/α2(IV) chains BM/stroma	α3(IV) chain BM/stroma	α4(IV) chain BM/stroma	α5(IV) chain BM/stroma	α6(IV) chain BM/stroma
Normal tissues	5	+ /+	−/−	−/−	+/−	+/−
Benign tumors						
Fibroadenoma	5	+ /+	−/−	−/−	+/−	+/−
Intraductal papilloma	6	++ /+	−/−	−/−	++/−	++/−
Malignant tumors	20					
Intraductal components of invasive ductal carcinoma		+ /+	−/−	−/−	+/−	+/−
Invasive ductal carcinoma		−~+(dc) /+	−/−	−/−	−/−	−/−

* Arbitrary grades for immunostaining intensity of α1(IV), α2(IV), α3(IV), α4(IV), α5(IV), and α6(IV) chains: negative, −; negative or discontinuous positive, −~+(dc); positive, +; strongly positive, ++.

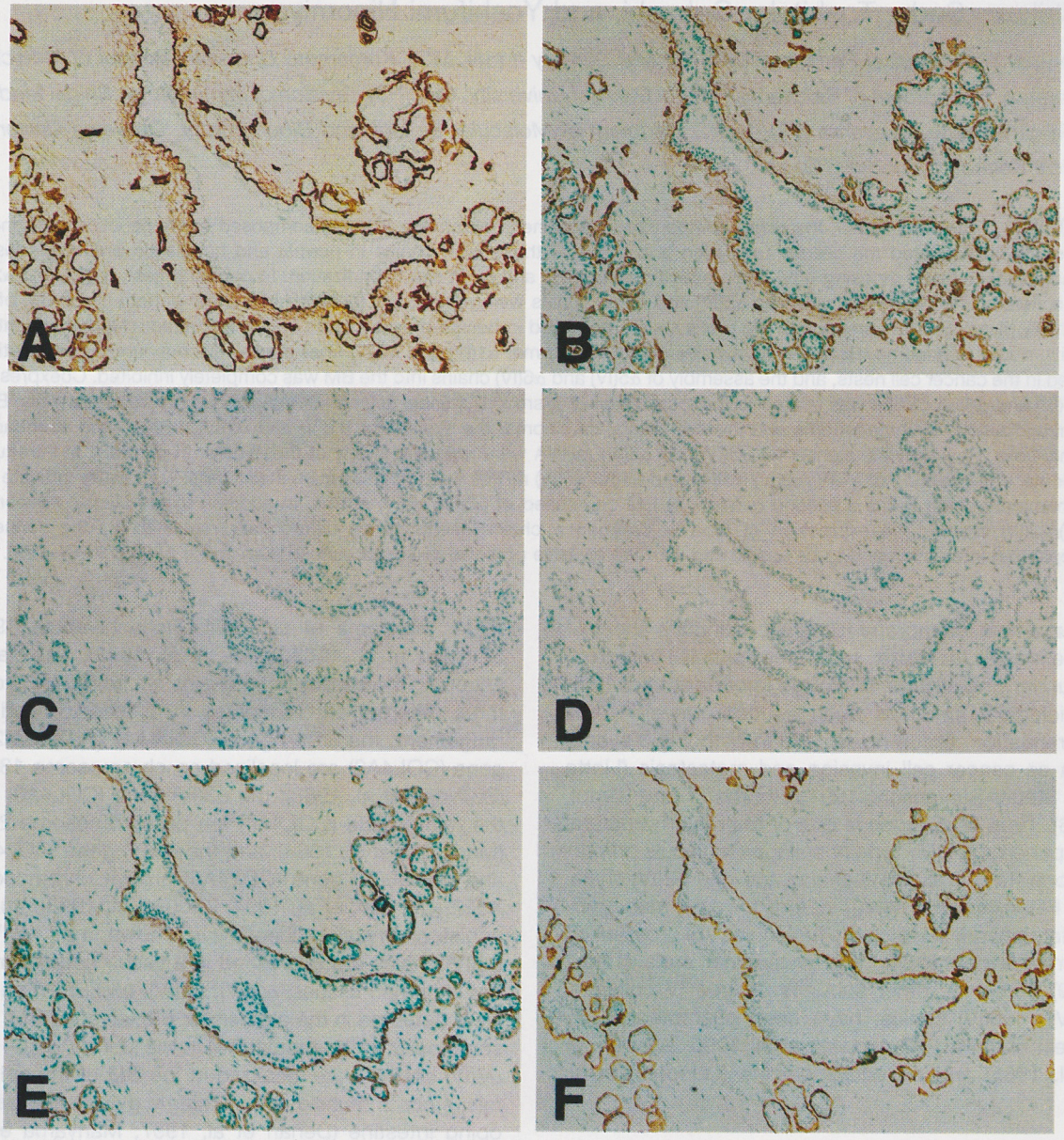


Figure 1.
Immunohistochemical localization of six different α(IV) chains in normal breast. Serial sections were stained with antibodies to α1(IV) (A), α2(IV) (B), α3(IV) (C), α4(IV) (D), α5(IV) (E), and α6(IV) (F) chains. A and B, α1(IV) and α2(IV) chains are stained in the basement membrane (BM) of mammary ducts and lobules, capillaries, and fibroblastic cells. E and F, α5(IV) and α6(IV) chains are restrictively stained in the BM of mammary ducts and lobules. No obvious staining of α3(IV) and α4(IV) chains is noted in the BM. Original magnification, ×100.

myoepithelium situated directly on the BM. Several authors have observed the coimmunolocalization of BM components and α-SMA–positive myoepithelium in benign breast tumors, and found the absence of BM components or the disappearance of the myoepithelium to be an evident feature in invasive ductal carcinoma (Gusterson et al, 1982; Rudland et al, 1993; Tsubura et al, 1988). Hewitt et al (1997) examined the localization of the type IV collagen subunits in normal and neoplastic breast tissues; however, there is little information about the precise expression of type IV collagen α chains in association with the localization of epithelial and myoepithelial cells evaluated by α1(IV) - α6(IV) chain-specific monoclonal antibodies and α-SMA antibody. Furthermore, the cellular origin of these type IV collagen α chains is unknown. It has been assumed that mammary epithelial cells synthesize their own BM (Streuli and Bissell, 1990; Warburton et al, 1982); however, in situ hybridization studies indicate that the signal for α1(IV) chain is present in mammary stromal cells, but not in mammary epithelial cells (Keely et al, 1995; Soini et al, 1994).

In the present study, we used immunohistochemistry combined with in situ hybridization to investigate the regulated expression and distribution of α1(IV), α2(IV), α5(IV), and α6(IV) chains in normal breast and in benign and malignant tumors. In addition, we examined the localization of α-SMA-positive myoepithelial cells and CD34-positive capillary endothelial cells in association with the differential expressions of type IV collagen α chains.

Results

Immunohistochemical Findings

The immunohistochemical results are summarized in Table 1. In normal breast, α1(IV) and α2(IV) chains were stained in a continuous linear pattern around the ducts and the lobules in mammary gland. In the periductal stroma, the linear immunostaining of α1(IV) and α2(IV) chains was also detected around capillaries and fibroblastic cells (Fig. 1, A and B). α5(IV) and α6(IV) chains were restrictively expressed in the BM in a clear single line around the ducts and the lobules, whereas α5(IV) and α6(IV) chains were not seen around capillaries and fibroblastic cells (Fig. 1, E and F). On the other hand, no evidence of positive staining for α3(IV) and α4(IV) chains was observed in the BM of the mammary glands (Fig. 1, C and D). Similar immunostaining patterns were confirmed in benign neoplasms, including fibroadenoma (Fig. 2, A to D) and intraductal papilloma (Fig. 3, A to D). Particularly, in the intraductal papilloma, the immunoreactivity of both α5(IV) and α6(IV) chains was higher than in other breast neoplasms (Fig. 3, C and D). In the intraductal

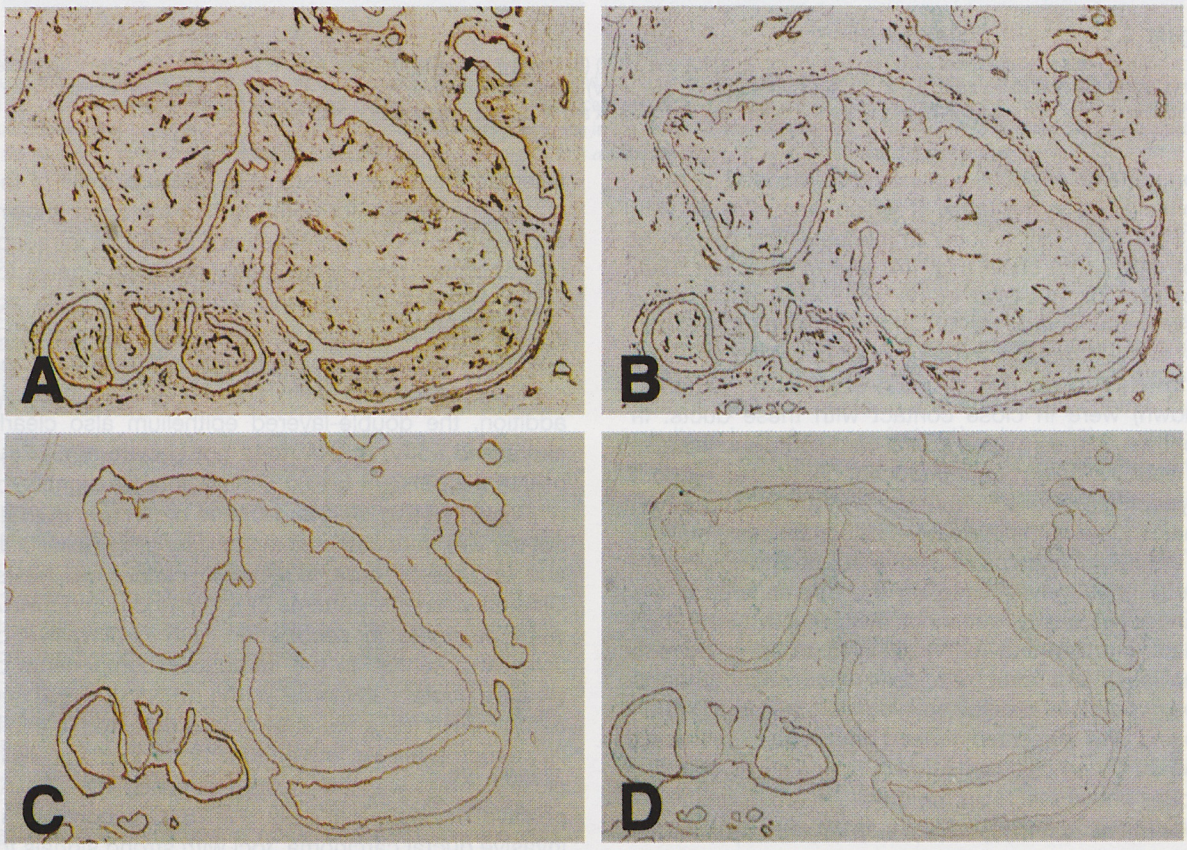


Figure 2.
Immunohistochemical localization of α1(IV) (A), α2(IV) (B), α5(IV) (C), and α6(IV) (D) chains in fibroadenoma. A and B, α1(IV) and α2(IV) chains are stained in the BM of elongated ducts and stromal cells including capillaries. C and D, α5(IV) and α6(IV) chains are restrictively stained as a single line in the BM of the elongated ducts in the serial sections of A and B. Original magnification, ×40.

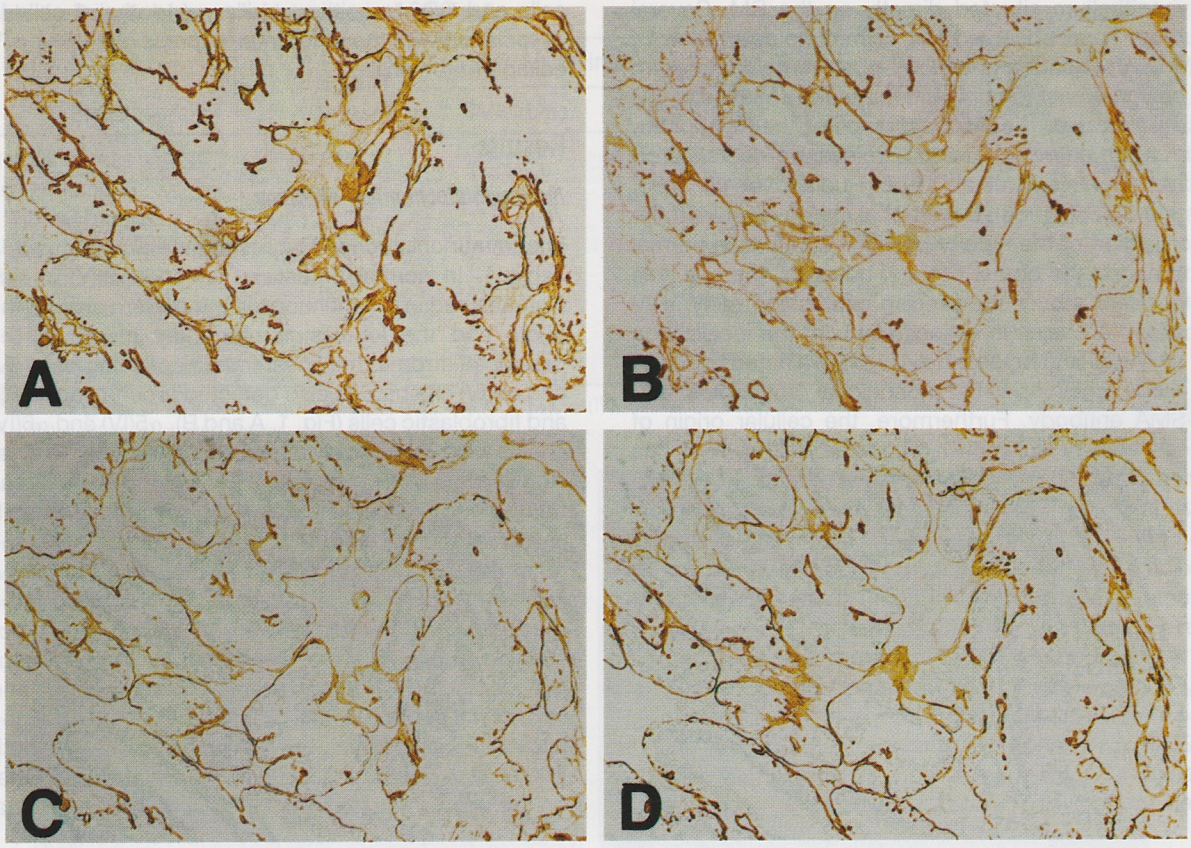


Figure 3.
Immunohistochemical localization of α1(IV) (A), α2(IV) (B), α5(IV) (C), and α6(IV) (D) chains in intraductal papilloma. A and B, α1(IV) and α2(IV) chains are stained in the BM of the proliferated ducts and in the stromal cells. α5(IV) and α6(IV) chains are restrictively stained as a single line in the BM. C and D, Strong immunoreactivity of α5(IV) and α6(IV) chains is noted in the serial sections of A and B. Original magnification, ×100.

components of ductal carcinoma surrounded by myoepithelial cells, the latter of which were stained with α-SMA (Fig. 4B), a linear immunostaining pattern of not only α1(IV) chain (Fig. 4A) and α2(IV) chain (data not shown), but also α5(IV) and α6(IV) chains was clearly identified in the BM of the ducts (Fig. 4, C and D). In the stroma, a large number of small capillaries with α1(IV) chain (Fig. 4A) and α2(IV) chain (data not shown) were in close contact with these ducts. In contrast, in the invasive ductal carcinoma and in areas of microinvasion of intraductal carcinoma without myoepithelial cells, α1(IV) chain (Fig. 5A) and α2(IV) chains (data not shown) were discontinuously stained in the marginal areas of the cancer cell nests or were partly negatively stained. In the stroma around the cancer cell nests, immunostaining reactivity of α1(IV) chain (Fig. 5A) and α2(IV) chain (data not shown) in capillaries and fibroblastic cells was evident. In addition, complete loss of staining of α5(IV) and α6(IV) chains was observed around the cancer cell nests (Fig. 5, C and D).

Localization of α1(IV), α2(IV), α5(IV), and α6(IV) mRNA

In fibroadenoma, signals for α1(IV) mRNA (Fig. 6C) and α2(IV) mRNA (data not shown) were evident in the CD34-positive capillary endothelial cells and fibroblastic cells around the mammary ducts (Fig. 6B), whereas there was no signal in epithelial cells of the ducts. The signals for α5(IV) mRNA (data not shown) and α6(IV) mRNA were negative (Fig. 6D). In intraductal papilloma, there were highly elevated signals for α1(IV) mRNA (Fig. 6G) and α2(IV) mRNA (data not shown) in the double-layered epithelium composed of epithelial cells and myoepithelial cells, and in the capillary endothelial cells and fibroblastic cells. In addition, the double-layered epithelium also clearly expressed α5(IV) mRNA (data not shown) and α6(IV) mRNA (Fig. 6H). In breast cancer, intense signals for α1(IV) mRNA (Fig. 6, K and O) and α2(IV) mRNA (data not shown) were detected in capillary endothelial cells and fibroblastic cells, but not in cancer cell nests. Differential spatial patterns of α1(IV) and α2(IV) mRNA were observed in association with noninvasive or invasive potential. In the intraductal components of invasive ductal carcinoma, α1(IV) mRNA (Fig. 6K) and α2(IV) mRNA (data not shown) was detected in capillary endothelial cells and periductal fibroblastic cells in close contact with the ducts delineated by α-SMA–positive myoepithelial cells (Fig. 6J). In contrast, in invasive ductal carcinoma, foci with strong signals for α1(IV) mRNA (Fig. 6O) and α2(IV) mRNA (data not shown) were distributed randomly in the stroma, but not seen in cancer cell nests. The signals for α5(IV) mRNA (data not shown) and α6(IV) mRNA (Fig. 6P)

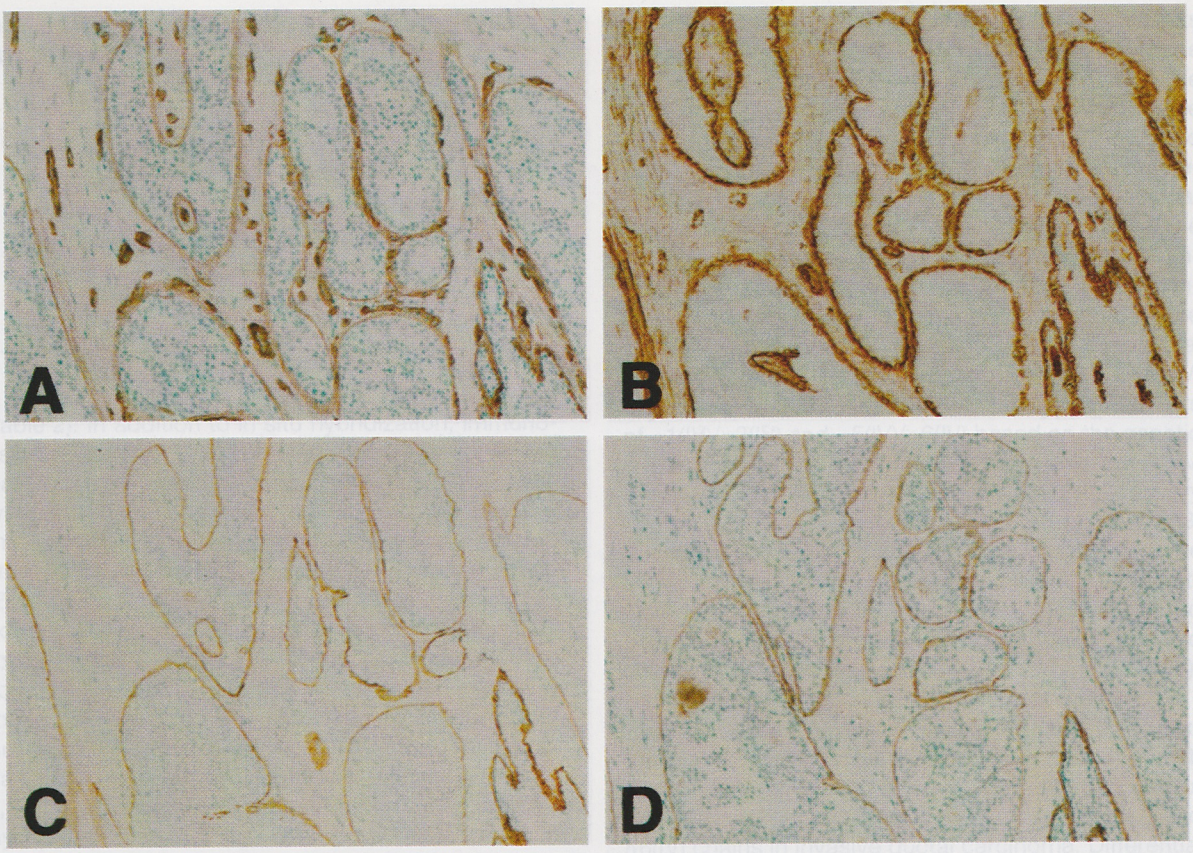


Figure 4.
Immunohistochemical localization of α1(IV) chain (A), α-SMA (B), α5(IV) (C), and α6(IV) (D) chains in the intraductal components of invasive ductal carcinoma. A, α1(IV) chain is stained in the BM surrounding the ducts replaced by cancer cells and in the capillary BM in close contact with the duct. C and D, the linear immunostaining of α5(IV) and α6(IV) chains is clearly identified in the BM zone in the serial sections of A and B. B, α-SMA–positive myoepithelial cells are noted in the intraductal components, and capillary pericytes and fibroblastic cells with α-SMA are also localized in the stroma. Original magnification, ×100.

were completely absent in both cancer cell nests and the stroma.

Results of semiquantitative analysis of α1(IV), α2(IV), α5(IV), and α6(IV) mRNA in fibroadenoma, intraductal papilloma, intraductal components of invasive ductal carcinoma, and invasive ductal carcinoma are summarized in Table 2.

Discussion

During the process of tumor invasion and metastasis, cancer cells have to degrade both the epithelial BM and capillary BM (Liotta and Stetler-Stevenson, 1991). Fragmentation or loss of BM is a characteristic morphologic feature of invasive breast cancer observed in immunohistochemical studies by using non–α-chain–specific type IV collagen antibodies (Albrechtsen et al, 1986; Barsky et al, 1983; Guelstein et al, 1993; Wetzels et al, 1989). The current study is the first to evaluate the expressions of α5(IV) and α6(IV) chains and their mRNA in breast tissues. As summarized in Table 1, colocalization of α5(IV) and α6(IV) chains was evident in the BM of mammary ducts in normal breast and in benign tumors and the intraductal components of invasive ductal carcinoma, all of which also expressed the classic type of α1(IV) and α2(IV) chains. However, α5(IV) and α6(IV) chains were absent both in the capillary BM and around fibroblastic cells. Interestingly, the assembly of α5(IV) and α6(IV) chains into the BM was completely inhibited in invasive ductal carcinoma. These results suggest that the discontinuous BM materials around the cancer cell nests originated from fibroblastic cells with expression of classic type of α(IV) chains, ie, α1(IV) and α2(IV) chains. Our result on breast cancer indicating loss of the expression of α5(IV) and α6(IV) chains is consistent with recent reports on basal cell carcinoma (Tanaka et al, 1997) and prostate carcinoma (Dehan et al, 1997). In addition, because coexpression of α5(IV) and α6(IV) chains was clearly related to the localization of α-SMA-positive myoepithelial cells, α5(IV) and α6(IV) chain–specific monoclonal antibodies may contribute to differentiate benign lesion with pseudoinvasion (eg, sclerosing adenosis) from invasive cancer (eg, tubular carcinoma) as a critical marker.

As shown in Table 2, in breast cancer, the signals for α1(IV) and α2(IV) mRNA found by in situ hybridization were evident mainly in the capillary endothelial cells and fibroblastic cells, not in the cancer cells. In this study, the lack of expression of α1(IV) and α2(IV) mRNA in the normal epithelial cells and cancer cells seems to be related to the sensitivity of the in situ hybridization method, to long half-life times of BM

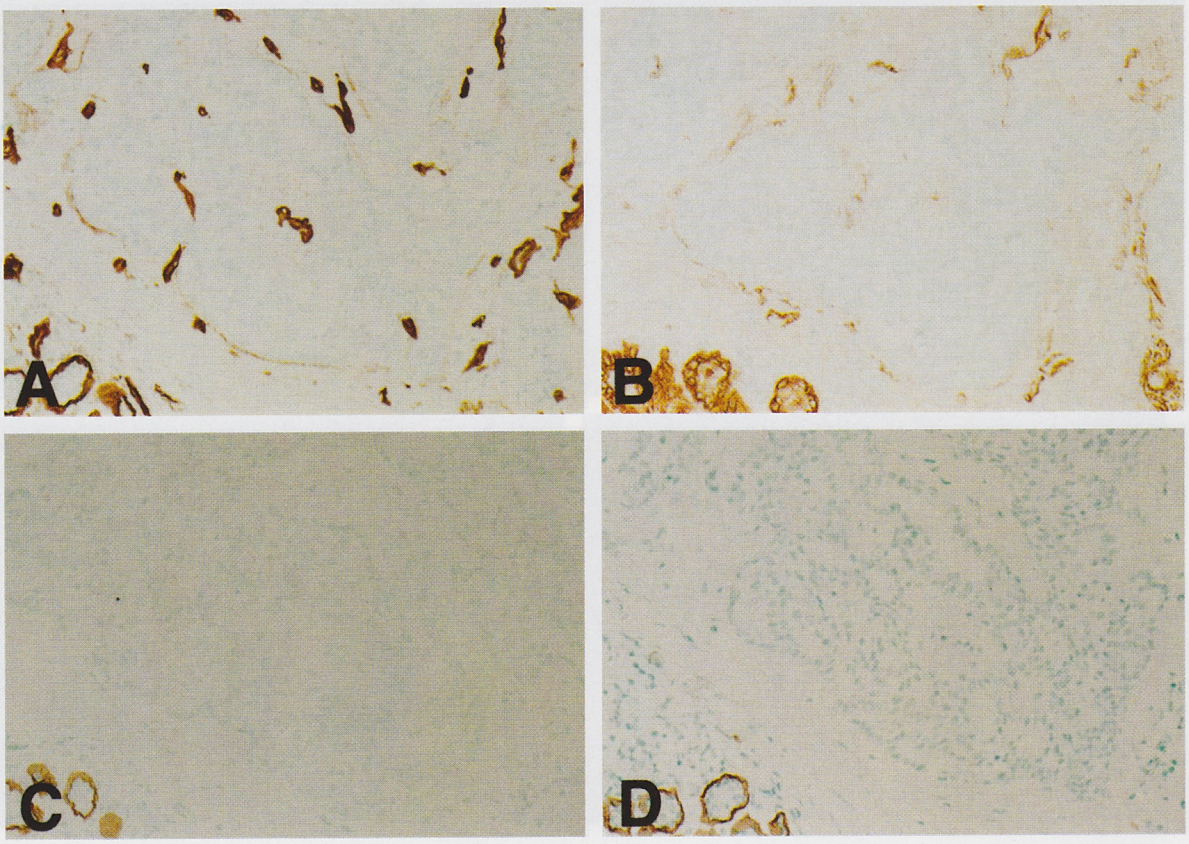


Figure 5.
Immunohistochemical localization of α1(IV) chain (A), α-SMA (B), α5(IV) (C), and α6(IV) (D) chains in invasive ductal carcinoma. A, α1(IV) chain is discontinuously stained in the marginal areas of the cancer cell nests or are partly negatively stained. C and D, complete loss of α5(IV) and α6(IV) chains is noted in the serial sections of A and B. B, α-SMA–positive myoepithelial cells are not seen around the invasive cancer cell nests, unlike in Figure 4B. A faint staining of α-SMA shows the localization of capillary pericytes and fibroblastic cells. Myoepithelial cells of normal mammary glands are observed at the lower right, as a positive control. Original magnification, ×200.

Table 2. Results of *In Situ* Hybridization Studies*

Breast disease	Number of cases	α1(IV)/α2(IV) mRNAs		α5(IV) mRNA		α6(IV) mRNA	
		Epithelium i/o	Stroma c/f	Epithelium i/o	Stroma c/f	Epithelium i/o	Stroma c/f
Normal tissues	5	−/−	−/−	−/−	−/−	−/−	−/−
Benign tumors							
Fibroadenoma	5	−/−	++/++	−/−	−/−	−/−	−/−
Intraductal papilloma	6	+/+	+/+	+/+	−/−	+/+	−/−
Malignant tumors	20						
Intraductal components of invasive ductal carcinoma		−/−	++/++§	−/−	−/−	−/−	−/−
Invasive ductal carcinoma		−	++/++‡	−	−/−	−	−/−

* Arbitrary grades of autoradiographic silver grains for α1(IV), α2(IV), α5(IV), and α6(IV) chain mRNA: −, negative (0 to 10 silver grains per cell); +, mild to moderate positive (10 to 20 silver grains per cell); ++, strongly positive (>20 silver grains per cells). §, ring-like pattern; ‡, random pattern. i, inner epithelial cells; o, outer myoepithelial cells; c, capillary endothelial cells; f, fibroblastic cells.

metabolism, or to loss of the gene expression in these epithelial cells. This evidence is consistent with several reports on colorectal cancer (Hewitt et al, 1992), lung cancer (Soini et al, 1993), and breast cancer (Soini et al, 1994). Furthermore, highly elevated signals for α1(IV) and α2(IV) mRNA were detected in the capillaries and fibroblastic cells in close contact with the intraductal components of invasive ductal carcinoma or areas of microinvasion. Indeed, Bose et al (1996) observed that periductal angiogenesis with a ring-like pattern in the intraductal carcinoma by immunohistochemical study by using factor VIII and CD34 antibodies. Unexpectedly, in intraductal papilloma, which is a benign tumor, the signals for not only α1(IV) and α2(IV) mRNA, but also those for α5(IV) and α6(IV) mRNA were identified in the mammary epithelial cells

Table 3. Histological Diagnosis of the Breast Tissues Used in this Study

Breast disease	Number of cases
Normal mammary gland	5
Fibroadenoma	5
Intraductal papilloma	6
Invasive ductal carcinoma with a predominant intraductal component	4
Invasive ductal carcinoma	16

(Table 2). In addition to in situ hybridization, immunohistochemistry by using α-SMA antibodies confirmed that both inner epithelial cells and myoepithelial cells synthesize α5(IV) and α6(IV) chains (Fig. 6, F, G, and H and Table 2). To our knowledge, there is no other in situ hybridization data about α1(IV), α2(IV), α5(IV), and α6(IV) mRNA expressions in intraductal papilloma. The expression of these α chains seems to be regulated by epithelial-myoepithelial interaction in intraductal papilloma. Recently α6(IV) mRNA was detected in cells of nonepithelial origin, fetal esophageal myocytes of muscular layers (Heidet et al, 1997) and dermal fibroblasts by using an in vitro skin model (Fleischmajer et al, 1997). Our results and these reports suggest that myoepithelial cells with α6(IV) gene expression are originated from the common ancestor of myocytes or fibroblasts.

In the intraductal components of invasive ductal carcinoma, capillary endothelial cells and periductal fibroblastic cells around the cancer cell nests actively synthesized α1(IV) and α2(IV) chains with a ring-like pattern. Similarly, Wapnir et al (1996) detected α1(I) and α1(III) procollagen transcripts in proximity to the BM zone as a ring-like pattern in ductal carcinomas in situ. These periductal stromal cells seem to take part in remodeling of the extracellular matrix including BM by various proteolytic enzymes. In in situ hybridization findings, we could show the possibility that newly formed BM composed of α1(IV) and α2(IV) chains secreted by stromal cells plays an important role as one of the barrier system against the activation of proteolytic enzyme and the matrix degradation. However, further studies are required to verify this hypothesis of BM remodeling and barrier system around cancer cell nests.

The most widespread BM type IV collagen consists of α1(IV) and α2(IV) chains. Two α1(IV) chains and one α2(IV) chain form triple-helical molecules, and these molecules organize the supramolecular network of the BM. However, the precise combination of α3(IV) to α6(IV) chains for construction of triple-helical molecules is still unknown. On the basis of an analysis that used pseudolysine, Kahsai et al (1997) reported the presence of two type IV collagen networks, one composed of α1(IV) - α6(IV) chains and the other composed of α3(IV) - α6(IV) chains, in addition to the classic network of α1(IV) and α2(IV) chains. Sado et al (1998) reported the presence of only α1(IV)/α2(IV) molecules, α3(IV)/α4(IV)/α5(IV) molecules, and others of α1(IV)/α2(IV) and α5(IV)/α6(IV) based on the genetic analysis and observations of the kidney and skin BM of patients with Alport syndrome, which is a hereditary nephritis caused by gene mutation of COL4A3, COL4A4, and COL4A5. Our results emphasize that the BM of mammary ducts contains two different networks of type IV collagen: the classic network formed by α1(IV)/α2(IV) chains, which is of stromal origin, and a second network formed by α1(IV)/α2(IV) and α5(IV)/α6(IV) chains, which is of epithelial origin.

In conclusion, this study demonstrated the coexpression of α5(IV) and α6(IV) chains in normal breast, benign tumors, and the intraductal components of invasive ductal carcinoma, and the loss of α5(IV) and α6(IV) chains in invasive ductal carcinoma. Differential expression of type IV collagen α chains of the mammary duct may be associated with the invasive potential of breast cancer.

Materials and Methods

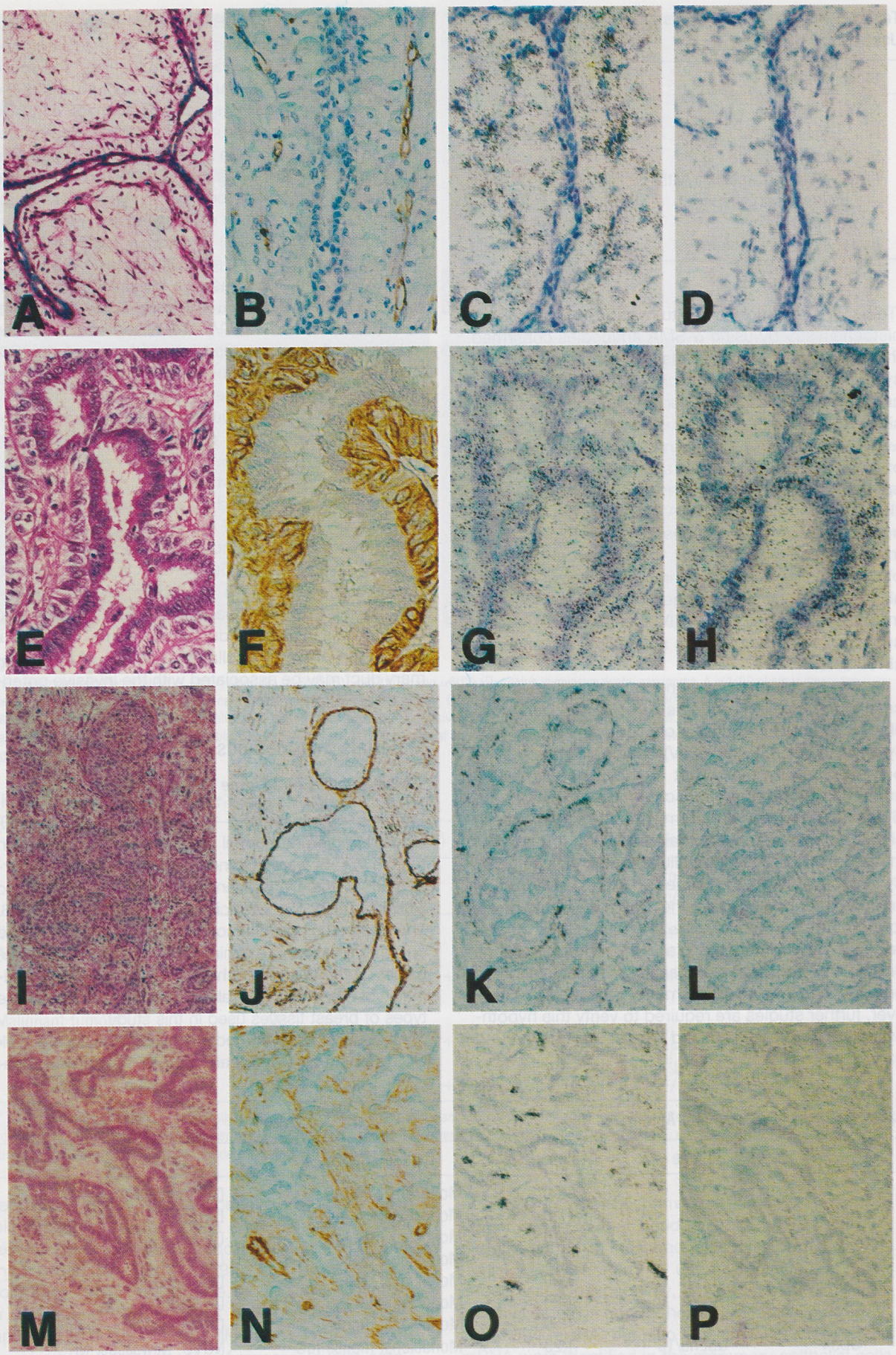
Tissue Samples and Tissue Preparation

Samples of benign ($n = 11$) and malignant ($n = 20$) breast tumors and normal breast tissue around benign tumors ($n = 5$) were obtained by surgical resection performed at the Department of Surgery II, Kumamoto University School of Medicine, between 1995 and 1998. The histopathologic diagnosis of the tumors was based on light microscopic examination according to the criteria set by the World Health Organization (1981) classification of breast tumors. Table 3 gives an overview of the types of breast tissues used in this study.

All tissues were immediately frozen in Tissue-Tek OCT compound (Miles Laboratories, Illinois) with ace-

Table 4. Panel of Monoclonal Antibodies Used

Specificity	Clone	Dilution	Type of antibody	Source
α1(IV)	H11	1:50	Monoclonal rat IgG	Dr. Sado, Okayama, Japan
α2(IV)	H21	1:50	Monoclonal rat IgG	Dr. Sado
α3(IV)	H31	1:10	Monoclonal rat IgG	Dr. Sado
α4(IV)	H43	1:10	Monoclonal rat IgG	Dr. Sado
α5(IV)	H52	1:20	Monoclonal rat IgG	Dr. Sado
α6(IV)	H63	1:30	Monoclonal rat IgG	Dr. Sado
α-SMA	1A4	1:50	Monoclonal mouse IgG	DAKO, Glostrup, Denmark
CD34	QBEnd/10	1:25	Monoclonal mouse IgG	NOVO, Newcastle, UK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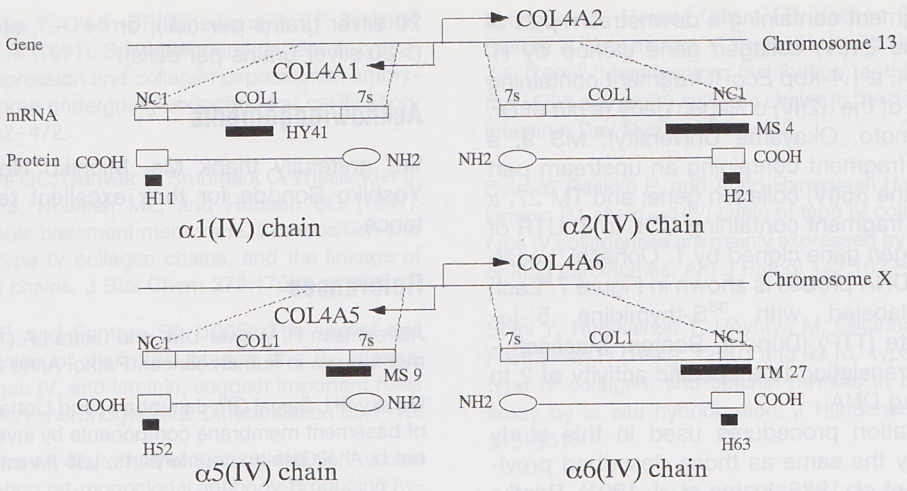

Figure 7.
Genomic organization of COL4A1/COL4A2 and COL4A5/COL4A6, and location of each of the cDNA probes (HY41, MS 4, MS 9,TM 27). α chain–specific monoclonal antibodies (H11, H21, H52, H63) are directed against the epitopes of the NC1 domains of these molecules. COL4A1 and COL4A2 are arranged head-to-head in close proximity on chromosome 13q34, and COL4A5 and COL4A6 on chromosome Xq22 are in a similar array.

tone/dry ice after extirpation for immunostaining of type IV collagen α chains and α-SMA. Several samples were fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4) (TAAB, EM Grade, United Kingdom) for 2 hours for CD34 immunostaining and in situ hybridization. After dehydration, they were embedded in paraffin.

Antibodies

Monoclonal antibodies to the six different α chains of human type IV collagen (H11, H21, H31, H43, H52, H63), α-SMA, and CD34 used in this study are shown in Table 4. Antibodies for α1(IV) - α6(IV) chains recognizing the sequences near the C-terminus of each NC1 domain were established by the rat lymph node method (Kishiro et al, 1995; Sado et al, 1995). According to epitope mapping multipin-peptide scanning, H11 recognizes the amino acid sequence KKPTPSTL; H21, DTLKAGLIR; H31, IPSTVKA; H43, PAPDTLKE; H52, SKPQSETL; and H63, GELP. The epitopes of the NC-1 domain of these antibodies are shown in Figure 7 (epitopes of H31 and H43 are not shown). α-SMA (clone 1A4), and CD34(clone QBEnd/10) were purchased from DAKO (Glostrup, Denmark) and NOVO (Newcastle, United Kingdom), respectively.

Immunohistochemistry

For immunostainings of α1(IV) - α6(IV) chains and α-SMA, 5-μm cryosections were fixed with acetone for 10 minutes. The sections stained for α1(IV) - α6(IV) chains were treated with 6 M urea (Gibco BRL, Maryland) in 0.1 M glycine-HCl buffer (pH 3.5) for 20 minutes at room temperature to linearize the antigens related to the synthetic peptides. For CD34 staining, 5-μm sections were deparaffinized. These sections were blocked for endogenous peroxidase with 1% H_2O_2 in methanol for 30 minutes and then washed in PBS. Some sections for CD34 immunostaining were trypsinized for 10 minutes with 0.5% calcium chloride buffer (pH 7.6). Thereafter, sections were immersed in 5% normal rabbit or horse serum in PBS for 30 minutes, covered with the primary antibody solution, and incubated overnight at 4° C. The optimal dilution of each primary antibody is shown in Table 4. The immunoreaction was performed by using a Vectastain peroxidase ABC kit (Vector Laboratories, Burlingame, California). Sections were washed in PBS, and the antigenic sites were demonstrated by reacting the sections with a mixture of 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Dojin Chemical, Tokyo, Japan) in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.01% H_2O_2 for 7 minutes. After washing in distilled water, the nuclei were stained with methyl green, and then the sections were dehydrated in ethanol, cleared in xylene, and mounted in Permount (Fisher Scientific, Fair Lawn, New Jersey).

In Situ Hybridization

For in situ hybridization, we used the following α(IV) chain–specific cDNA probes: HY41, a 811-base pair

Figure 6.
Expression of α1(IV) mRNA (C, G, K, O) and α6(IV) mRNA (D, H,L, P), and immunohistochemical localization of CD34 (B) and α-SMA (F, J,N) in benign and malignant neoplasms of the breast. A to D, in fibroadenoma, α1(IV) mRNA is detected in CD34-positive capillary endothelial cells and periductal fibroblastic cells, but is undetectable in the epithelial cells (C). E to H, in intraductal papilloma, α1(IV) mRNA is identified in both epithelial cells and α-SMA–positive myoepithelial cells (G). α6(IV) mRNA is also noted in both epithelial cells and myoepithelial cells (H) in a similar pattern as in G. I to L, in the intraductal components of invasive ductal carcinoma, strong signals for α1(IV) mRNA are detected as a ring-like pattern in capillary endothelial cells and periductal fibroblastic cells in close contact with the ducts delineated by α-SMA–positive myoepithelial cells (J, K). M to P, in the invasive ductal carcinoma, foci with intense signals for α1(IV) mRNA are randomly distributed in the stromal cells but are not seen in cancer cells (O). Except in intraductal papilloma, no obvious signals for α6(IV) mRNA are seen in the breast tissues (D, L,P). Left panels (A, E, I, M), hematoxylin and eosin stain. Original magnifications: ×100 in A, ×200 in B to E, ×280 in F to H, ×130 in I to L, ×200 in M to P.

(bp) *Eco*RI fragment containing a downstream part of the COL of the α1(IV) collagen gene cloned by H. Yoshioka; MS 4, a 1.4-kbp *Eco*RI fragment containing COL to 3′ UTR of the α2(IV) collagen gene (a gift of Dr. Manabu Sugimoto, Okayama University); MS 9, a 670-bp *Eco*RI fragment containing an upstream part of the COL of the α5(IV) collagen gene; and TM 27, a 1.8-kbp *Eco*RI fragment containing COL to 3′ UTR of the α6(IV) collagen gene cloned by T. Oohashi. Localization of the cDNA probes is shown in Figure 7. Each probe was labeled with ^{35}S-thymidine 5′-[α-thio]triphosphate (TTP) (Dupont, Boston, Massachusetts) by nick-translation to a specific activity of 2 to 4×10^8 cpm/μg DNA.

The hybridization procedures used in this study were essentially the same as those described previously (Hayashi et al, 1986; Iyama et al, 1991). Briefly, deparaffinized sections were treated with Pronase E (0.25 mg/ml in 50 mM Tris-HCl [pH 7.6] and 5 mM disodium EDTA [Sigma, St. Louis, Missouri]) for 10 minutes, and acetylated with a freshly diluted acetic anhydride (0.25% in 0.1 M triethanolamine buffer [pH 8.0]) for 10 minutes. The slides were washed twice in 2× standard saline-citrate buffer (SSC; 1× SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) for 5 minutes each, dehydrated in ethanol, and dried in air.

The treated sections were processed for in situ hybridization at 45° C for 18 hours in a mixture containing the tritiated cDNA probe (1 μg/ml), yeast tRNA (500 μg/ml), salmon sperm DNA (80 μg/ml), 50% formamide, 10 mM Tris-HCl (pH 7.0), 0.15 M NaCl, 1 mM EDTA (pH 7.0), 1× Denhardt's mixture, and 10% dextran sulfate.

After hybridization and removal of the cover glass by immersing the slides in 2× SSC for 1 hour at room temperature, sections were washed three times in 2× SSC for 10 minutes each at room temperature, once in 0.5× SSC for 10 minutes at 45° C, and three times in 0.1× SSC for 10 minutes each at 45° C. Afterward, the slides were dehydrated in ethanol, dried in air, immersed in Kodak NTB-2 emulsion, and exposed for 5 days at 4° C. The exposed slides were developed in Kodak D-19 developer for 3 minutes at 18° C. The sections were counterstained with hematoxylin.

Differential expression of each mRNA in comparable tissue sections provided a good internal control. In addition, sections digested with RNase (2 mg/ml, 1 hour at room temperature) before in situ hybridization with the cDNA probe showed no labeling, suggesting that the hybridization with the probe was dependent on the presence of RNA in tissue sections.

After autoradiography was performed, signals for α1(IV), α2(IV), α5(IV), and α6(IV) mRNA were analyzed separately in tumor cells and stromal cells (endothelial cells and fibroblastic cells) in the fibroadenoma, intraductal papilloma, and invasive ductal carcinoma. The number of autoradiographic silver grains per cell was quantified by duplicate counting (250× oil immersion) of 50 cells in each of five random fields. This process was repeated for each of three experiments. mRNA intensity was graded as "−", negative (0 to 10 silver grains per cell); "+", mild to moderate positive (10 to 20 silver grains per cell); or "+ +", strongly positive (>20 silver grains per cells).

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