Virchows Arch (1999) 435:496–500

ORIGINAL ARTICLE

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Involvement of tenascin-C in proliferation and migration of laryngeal carcinoma cells

Received: 5 March 1999 / Accepted: 7 June 1999

Abstract Tenascin-C (TN-C) is an extracellular matrix glycoprotein upregulated in various pathological processes. In this study, we investigated its distribution in dysplasia and carcinoma of the human larynx using immunohistochemistry and in situ hybridization (ISH) techniques. In all cancer tissues, TN-C immunostaining was markedly increased in the stroma, especially around the cancer cell nests. In addition, cytoplasmic staining of cancer cells was also observed in 62.5% of the invasive cases, the cells being distributed in the periphery of the nests adjacent to the stroma. TN-C mRNA signals in cancer cells were detected in all six cases examined by ISH. Furthermore, in vitro evaluation of the roles of TN-C demonstrated an increase in the proliferating cell fraction in a dose-dependent manner. In a wound closure assay, the addition of TN-C promoted migration. We conclude that TN-C secreted by cancer cells may be involved in their proliferation and migration in an autocrine fashion.

Key words Tenascin-C · Laryngeal carcinoma · Immunohistochemistry · In situ hybridization · Cell proliferation · Cell migration

Introduction

Many immunohistochemical studies have demonstrated that tenascin-C (TN-C) is upregulated in cancer tissues. Although it has been considered that stromal cells are the major cellular origin of TN-C, recent ISH studies have demonstrated that epithelial cancer components can also act as a source [14, 19, 20]. During embryogenesis, epithelial expression of TN-C becomes apparent in a variety of tissues [10, 18], this being closely associated with cell proliferation and migration. Neoplastic epithelial cells might therefore be expected to exhibit reactivated TN-C expression, and immunohistochemistry has shown squamous cell cancers to bind anti TN-C antibody frequently in the cytoplasmic compartment [15, 17]. In this study, we examined dysplasias and squamous cell carcinomas of human larynx by means of immunohistochemistry using anti-TN-C antibody and in situ hybridization (ISH) using digoxigenin-labeled cRNA probes for TN-C mRNA.

In addition, since in vitro studies have demonstrated that TN-C may promote mitogenesis and migration of some [2, 3, 6, 7], but not all cell lines in culture [4, 12, 16], we investigated its effects on proliferation and migration of epithelial cells of the human laryngeal cancer cell line HEp-2 in vitro.

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Materials and methods

Thirty-eight cases of human laryngeal cancer (6 of carcinoma in situ, 32 invasive cases) and five dysplastic lesions were investigated. Of the invasive cancer samples, 26 were obtained by means of biopsy, and 6 were surgically resected, including 5 after preoperative irradiation. Three normal tissue specimens were also obtained from autopsy cases. Archival samples were fixed with 10% formalin and routinely embedded in paraffin. For ISH, six freshly obtained samples were immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, at 4°C overnight; after being rinsed in PB, they were embedded in paraffin. All blocks were sectioned at 4 µm and placed on silane-coated glass slides (Dako Japan, Kyoto, Japan). Histological diagnosis was carried out using sections stained with hematoxylin and eosin.

The primary antibody for human TN-C, RCB1, was as previously described [9]. After deparaffinization with xylene and rehydration through graded ethanols, the sections were equilibrated in distilled water, and incubated in 0.4% pepsin (1:60,000 Sigma) in 0.01 N HCl for 20 min at 37°C to retrieve the antigens. The sections were incubated in 0.3% H_2O_2 in methanol for 15 min to block endogenous peroxidase activity. All sections were treated with super block solution (Scytek Laboratories, Logan, Utah) before incubation with TN-C antibody (0.1 µg/ml) overnight at 4°C. After washing, a commercially available LSAB kit (Scytek) and diaminobenzidine (DAB)/H_2O_2 solution were used to demonstrate

antibody binding. Light counterstaining with hematoxylin was performed to aid orientation.

For ISH, human TN-C cDNAs were a generous gift from Dr. L. Zardi (Genoa, Italy). Preparation of digoxigenin-labeled TN-C cRNA probes and performance of ISH were achieved using previously described methods [9, 20]. ISH signals were detected immunohistochemically with alkaline-phosphatase-conjugated anti-DIG antibody, with visualization by incubation in a nitrotetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution. The sections were counterstained with 0.1% nuclear fast red in 5% aluminum sulfate solution and air-dried before mounting.

TN-C was purified from culture supernatant of U251 MG human glioma cells U-251 MG by ammonium sulfate precipitation, Sephacryl S-500 gel filtration, and Mono Q ion-exchange chromatography as previously described [1].

The laryngeal cancer cell line, HEp-2, was maintained in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS, Gibco-BRL, Grand Island, N.Y.) at 37°C under a 5% CO_2 atmosphere. For BrdU incorporation assays, the cells were grown on fibronectin-coated culture slides (Becton Dickinson, Bedford, Mass.), washed, and incubated in DMEM with 0.1% FCS for 24 h. Then, TN-C was added into the medium at a final concentration of 10 µg/ml or 20 µg/ml. After incubation for 12 h, the cells were labeled with 8-bromodeoxyuridine (BrdU 10 µg/ml) for 2 h. Cells were fixed with 70% ethanol at –20°C for 30 min and treated with 1 *N* HCl solution at room temperature for 20 min. Labeled nuclei were detected with monoclonal anti-BrdU antibody (DAKO Japan, Kyoto, Japan) and secondary anti-mouse IgG antibody conjugated with peroxidase (MBL, Nagoya, Japan), followed by color development in DAB/H_2O_2 solution. The nuclei were lightly counterstained by methylgreen. The percentage of nuclei that were labeled was used as a cell-proliferation index. The given values are the means of 16 determinations from four individual experiments.

Wound migration assays were performed by the method of Chung et al. [3]. Cells (1×10^6) were plated into each well of fibronectin-coated 12-well plates, and grown to confluence. Twenty four hours before the assay, the cells were washed and incubated in DMEM with 0.1% FCS. The confluent monolayers were scraped with a micropipette tip, washed, and examined on films under a phase-contrast microscope. TN-C was then added into the medium at a final concentration of 20 µg/ml. Twelve hours later, the same scraped areas were examined again. Cell migration was determined by measuring the distance between the wound edges in the photographs at 0 h and 12 h. The values are the means for 15 or more fields from three independent cultures.

Results

In normal laryngeal tissues, TN-C was immuno-labeled in a thin layer beneath the mucosal epithelia (Fig. 1a). In dysplastic lesions, TN-C labeling under the epithelial layer became broader and more intense (Fig. 1b). This was even more prominent in carcinoma in situ (CIS) (Fig. 1c), although cytoplasmic staining of cancer cells was not observed. In invasive cancer tissues, all cases showed marked deposition of TN-C in the stroma (Fig. 1d), particularly around the cancer nests. In addition, cytoplasmic staining of the cancer cells was observed in 20 of 32 cases (62.5%), usually in cells distributed in the basal layer of the intraepithelial lesions projecting into the subepithelial connective tissues and in the periphery of the invasive nests. ISH demonstrated cancer cell expression of TN-C mRNA in all of the six cases examined (Fig. 1e). The labeled cells were located in the peripheral zone adjacent to the stroma, as noted for cytoplasmic immuno-labeling.

Thus, cancer cells of laryngeal squamous cell carcinomas were found to produce and secrete TN-C frequently, in association with invasion into the subepithelial connective tissues.

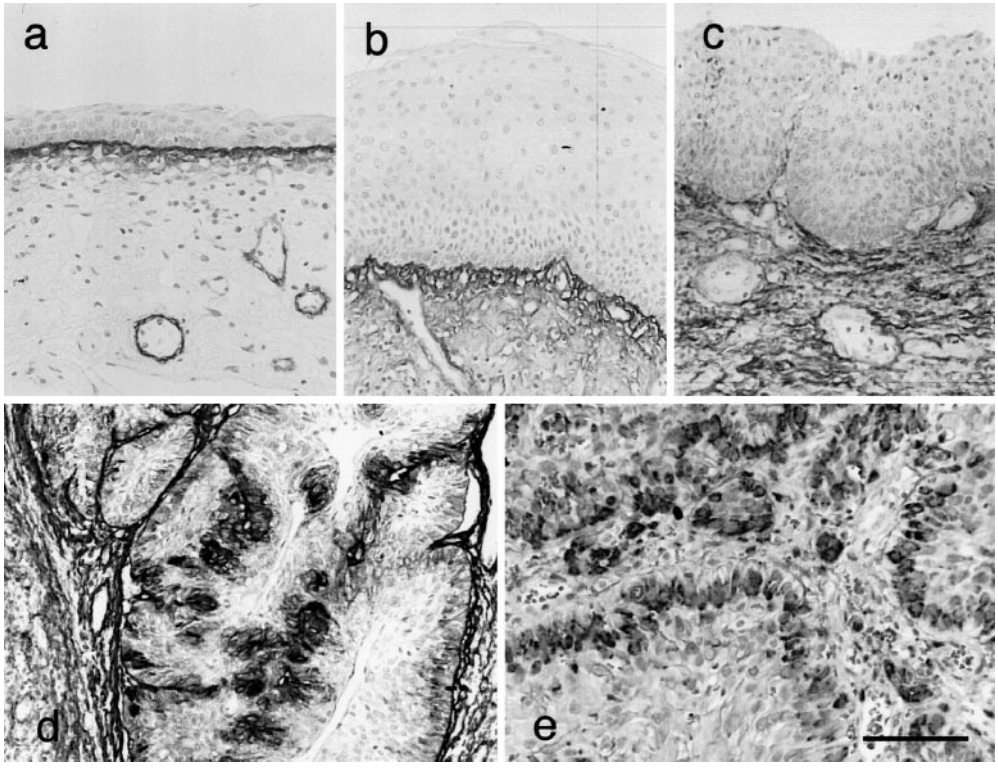

Fig. 1 Localization of tenascin-C (TN-C) protein (a–d) and its transcripts (e) in normal and neoplastic lesions of the larynx. **a** TN-C is immuno-labeled in a thin layer beneath normal mucosal epithelium. **b** TN-C labeling under the epithelial layer is broader and more intense in a dysplastic case. **c** TN-C immunostaining is broader and more intense in a carcinoma in situ, but cytoplasmic staining of the epithelial cells is not apparent. **d** Prominent deposition of TN-C in the stroma of an invasive cancer, especially around the cancer nests. The cytoplasm of the cancer cells is positively labeled, in the basal layers of the intraepithelial lesions projected into the subepithelial connective tissues. **e** In situ hybridization (ISH) for TN-C mRNA. Note the signals in cell in the peripheral zone adjacent to the stroma. *Bar* 100 µm

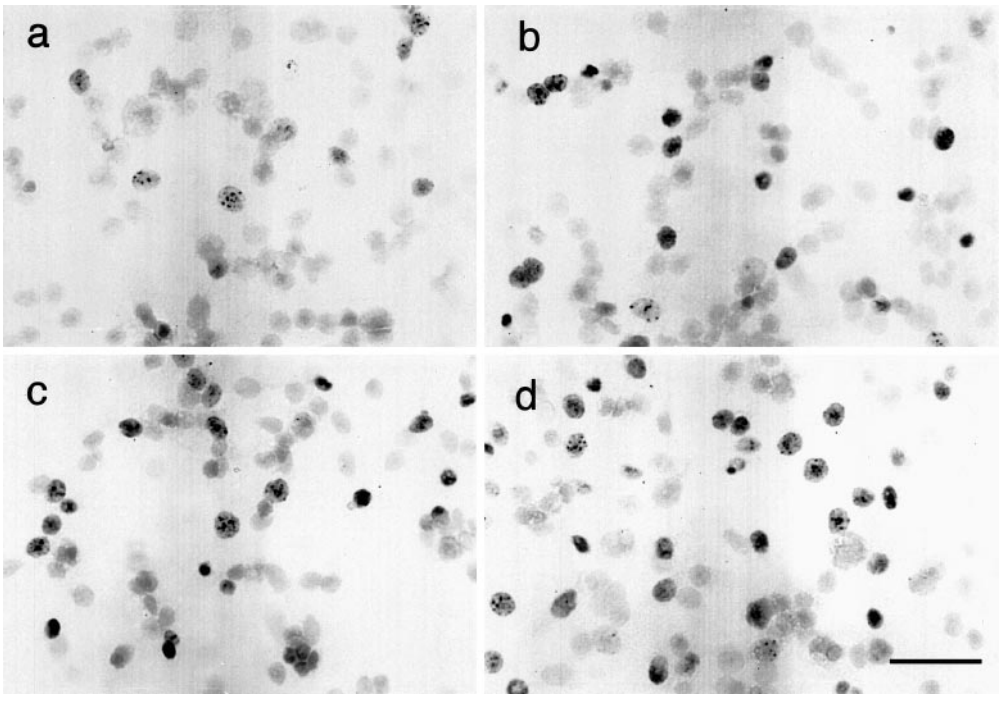

Fig. 2 8-Bromodeoxyuridine (BrdU) incorporation into HEp-2 cells after addition of tenascin-C (TN-C) into the medium. HEp-2 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) with 0.1% FCS for 24 h, and then incubated without **a** and with 10 µg/ml **b** and 20 µg/ml **c** TN-C in DMEM with 0.1% fetal calf serum (FCS). **d** A positive control incubated with medium supplemented with 10% FCS. *Bar* 50 µm

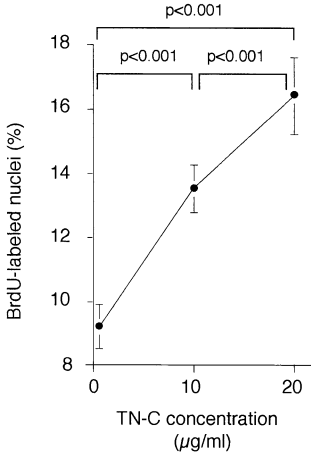

Fig. 3 Mitogenic activity of HEp-2 cells treated with tenascin-C (TN-C) as assessed by 8-bromodeoxyuridine (BrdU) incorporation assay. The percentages of labeled nuclei to total nuclei are shown. The values are the means of 16 determinations from four individual experiments. The differences are significant (P<0.001)

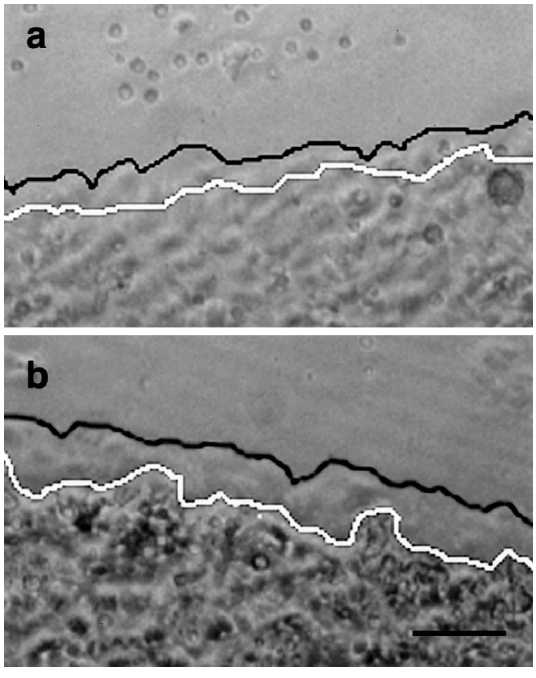

Fig. 4 The effects of tenascin-C (TN-C) on HEp-2 cell migration tested in an in vitro wound closure assay. No TN-C **a** or 20 µg/ml **b** TN-C were added after wounding. Photographs taken at 12 h after wounding were overlayed on photographs at 0 h. *White lines* indicate starting positions, and *black lines* are migrating fronts 12 h thereafter. *Bar* 100 µm

Laryngeal cancer cells, HEp-2, were chosen to clarify roles of TN-C in vitro, because of their lack of production of TN-C and fibronectin [11]. In preliminary studies, we used culture plates and slides without fibronectin coating. However, addition of TN-C into the medium induced rounding of the cells and detachment from the substratum, due to the counter-adhesive effects of TN-C. On fibronectin, the cells remained adherent after the TN-C treatment. Addition of TN-C induced increased cell proliferation in a dose-dependent manner. The number of nuclei labeled during a 2-h incubation with BrdU was 13.5±0.8% and 16.4±1.2% in the medium with 10 µg/ml and 20 µg/ml, respectively, of TN-C, while the figure in medium without TN-C was 9.2±0.7% (Fig. 2 and Fig. 3).

In in vitro wound closure assay, the migrated distance was 69.4±16.1 µm in the medium with 20 µg/ml TN-C, while in the medium without TN-C it was 53.0±14.2 µm

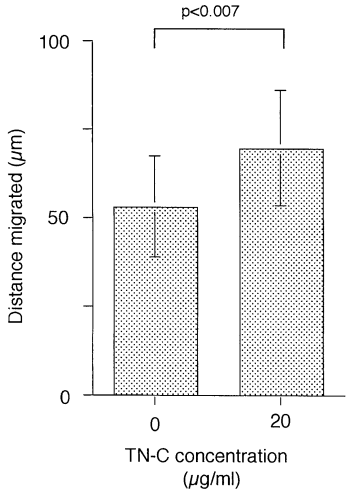

Fig. 5 Quantitative analysis of the wound closure assay. Cell migration was determined by measuring the distance between the wound edges in the photographs at 0 h and 12 h. The value are the means of 15 or more fields from three independent cultures. There is a significant difference (P<0.007) between with or without tenascin-C (TN-C)

(Fig. 4 and Fig. 5). The 31% increase was statistically significant (P<0.007).

Discussion

The present investigation showed that 62.5% of laryngeal carcinomas exhibited cytoplasmic staining. Previous immunohistochemical studies have demonstrated cytoplasmic staining of the tumor cells by anti-TN-C antibody in squamous cell carcinomas from various organs and tissues [15, 17]. The findings suggest that cancer cells themselves can produce and secrete TN-C. Indeed, our ISH showed cancer cell expression of TN-C mRNA in all six cases examined.

A recent ISH study on skin wound healing demonstrated TN-C mRNA in basal cells of the sheets of migrating keratinocytes 2–4 days after wounding, and in basally located keratinocytes of the neo-epidermis after 7 days [13]. It is considered that TN-C may facilitate keratinocyte migration by weakening the adhesion to fibronectin and other extracellular matrix proteins, allowing subsequent closing of the wound. During chick feather morphogenesis, TN mRNA is detected initially in the overlying epithelial cells, budding into the dermis, and then in the underlying mesenchyme [18]. We have also found that TN mRNA is expressed in the epithelial cells of mouse mammary buds on the 14th to 15th day of gestation [10]. In these processes, the epithelial cells extend their structures into the mesenchymal tissues, accompanied by cell proliferation. Thus, TN-C can be assumed to be closely linked to cell proliferation and migration in vivo.

Previous in vitro studies, however, have provided controversial results regarding the roles of TN-C in cell proliferation and migration. One study using NIH 3T3 cells demonstrated inhibition of cell proliferation on addition of TN-C [4], whereas another showed a promotive effect on the proliferation of the same cells [6]. Mammary tumor cells attached to TN-coated substrates and showed an increase in proliferation [2]. In contrast, TN-C addition inhibited proliferation of the mouse epidermal cell line Pam 212 [6]. More recently, it was demonstrated that TN-C has mitogenic activity in endothelial cells [3]. A similarly unclear situation also exists for cell migration. TN-C in the substratum stimulates migration of neural crest cells [7], but inhibits that of oligodendrocyte precursor cells [12]. When added to the medium, it inhibits mesodermal cell migration [16], while promoting endothelial cell migration [3]. In this study, we demonstrated promotive effects of addition of TN-C on both cell proliferation and migration of neoplastic epithelial cells cultured on fibronectin-coated substratum. TN-C and fibronectin are co-expressed in cancer tissues, such as breast and colon cancers [8, 20] and, in laryngeal cancer, fibronectin and TN-C are densely deposited in the basement membrane zone and in the stroma (data not shown). TN-C in tumor matrices, in collaboration with fibronectin, may provide a positive stimulus to proliferation and migration of cancer cells. In support of this conclusion, it has been reported that TN-C enhances migration of glioma cells on a fibronectin-coated substratum [5].

In conclusion, the present study provided evidence that TN-C produced and secreted by cancer cells could be involved in intraepithelial extension and invasion into the subepithelial connective tissues, by enhancing mitotic activity and migration of cancer cells themselves.

Acknowledgements We thank Ms. Satsuki Naota for her technical expertise and Dr. Malcolm A. Moore for his editorial assistance. This research was supported, in part, by Grants-in-Aids from the Ministry of Education, Science, Culture and Sports of Japan.

References

1. Aukhil I, Slemp CC, Lightner VA, Nishimura K, Briscoe G, Erickson HP (1990) Purification of hexabrachion (tenascin) from cell culture conditioned medium, and separation from a cell adhesion factor. Matrix 10:98–111
2. Chiquet-Ehrismann R, Mackie EJ, Pearson CA, Sakakura T (1986) Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. Cell 47:131–139
3. Chung CY, Murphy-Ullrich JE, Erickson HP (1996) Mitogenesis, cell migration, and loss of focal adhesions induced by tenascin-C interacting with its cell surface receptor, annexin II. Mol Biol Cell 7:883–892
4. Crossin KL (1991) Cytotactin binding: inhibition of stimulated proliferation and intracellular alkalinization in fibroblasts. Proc Natl Acad Sci U S A 88:11403–11407
5. Deryugina EI, Bourdon MA (1996) Tenascin mediates human glioma cell migration and modulates cell migration on fibronectin. J Cell Sci 109:643–652
6. End P, Panayotou G, Entwistle A, Waterfield MD, Chiquet M (1992) Tenascin: a modulator of cell growth. Eur J Biochem 209:1041–1051
7. Halfter W, Chiquet-Ehrismann R, Tucker RP (1989) The effect of tenascin and embryonic basal lamina on the behavior and morphology of neural crest cells in vitro. Dev Biol 132:14–25

8. Hanamura N, Yoshida T, Matsumoto E, Kawarada Y, Sakakura T (1997) Expression of fibronectin and tenascin-C mRNA by myofibroblasts, vascular cells and epithelial cells in human colon adenomas and carcinomas. Int J Cancer 73:10–15
9. Ishihara A, Yoshida T, Tamaki H, Sakakura T (1995) Tenascin expression in cancer cells and stroma of human breast cancer and its prognostic significance. Clin Cancer Res 1:1035–1041
10. Kalembey I, Yoshida T, Iriyama K, Sakakura T (1997) Analysis of tenascin mRNA expression in the murine mammary gland from embryogenesis to carcinogenesis: an in situ hybridization study. Int J Dev Biol 41:569–573
11. Kawakatsu H, Shiurba R, Obara M, Hiraiwa H, Kusakabe M, Sakakura T (1992) Human carcinoma cells synthesize and secrete tenascin in vitro. Jpn J Cancer Res 83:1073–1080
12. Kiernan BW, French-Constant C (1993) Oligodendrocyte precursor (O-2A progenitor cell) migration; a model system for the study of cell migration in the developing central nervous system. Dev Suppl, pp 219–225
13. Latijnhouwers M, Bergers M, Ponec M, Dijkman H, Andriessen M, Schalkwijk J (1997) Human epidermal keratinocytes are a source of tenascin-C during wound healing. J Invest Dermatol 108:776–783
14. Lightner VA, Marks JR, McCachren SS (1994) Epithelial cells are an important source of tenascin in normal and malignant human breast tissue. Exp Cell Res 210:177–184
15. Mori M, Muramatsu Y, Yamada K, Shrestha P, Takai Y (1996) Intracellular localization of tenascin in squamous cell carcinoma of oral cavity: an immunohistochemical study. Anticancer Res 16:3075–3079
16. Riou JF, Shi DL, Chiquet M, Boucaut JC (1990) Exogenous tenascin inhibits mesodermal cell migration during amphibian gastrulation. Dev Biol 137:305–317
17. Soini Y, Paakko P, Nuorva K, Kamel D, Linnala A, Virtanen I, Lehto VP (1993) Tenascin immunoreactivity in lung tumors. Am J Clin Pathol 100:145–150
18. Tucker RP (1991) The sequential expression of tenascin mRNA in epithelium and mesenchyme during feather morphogenesis. Rou'x Arch Dev Biol 200:108–112
19. Tuominen H, Pollanen R, Kallioinen M (1997) Multicellular origin of tenascin in skin tumors – an in situ hybridization study. J Cutan Pathol 24:590–596
20. Yoshida T, Matsumoto E, Hanamura N, Kalembeyi I, Katsuta K, Ishihara A, Sakakura T (1997) Co-expression of tenascin and fibronectin in epithelial and stromal cells of benign lesions and ductal carcinomas in the human breast. J Pathol 182: 421–428