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Original Article

The Biological Relevance of Laminin 5γ2 Expression at the Invading Edge of Colonic Carcinomas

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Abstract: Previous studies have shown the presence of dilated neoplastic glands with cellular gaps called glandular pores (GPs) and laminin 5γ2 expression at the invading edge of colonic carcinomas. We now extended our studies to explore a possible association between GP formation and laminin 5γ2 expression at the invading edge of colonic carcinomas. Immunostain was performed on sections of five consecutive neoplastic glands with and without GPs from 86 colonic adenocarcinomas to assess the expression of laminin 5γ2. Neoplastic glands with GPs were observed in 85% (73/86) of the tumors. Laminin 5γ2 was expressed in 92% (335/365) of the neoplastic glands with GPs but only in 17% (63/365) of the neoplastic glands without GPs ($p<0.05$). Laminin 5γ2 was overexpressed in the cells at the free ends of the pores in 88% of the neoplastic glands with GPs, but only in 14% of those without pores ($p<0.05$). Hence, at the growing edge of colonic carcinomas, laminin 5γ2 was frequently expressed in neoplastic glands having GPs. Remarkably, the tumor cells at the free ends of the GPs overexpressed laminin 5γ2, indicating increased production of this adhesion-migration macromolecule. The results suggest a close interaction between this adhesion-migration macromolecule, PG formation and the local progression of colonic carcinomas.
Key Words: Colorectal, adenocarcinomas, growing edge, pore formation, laminin 5γ2

Introduction

Laminins are a growing superfamily of large multidomain αβγ heterotrimetric glycoproteins [1-2]. They are the major cell substrates that regulate not only cell adhesion to the epithelial basement membrane (BM) but also normal cellular functions, such as proliferation, polarity and differentiation by interacting with integrins α6β1, α3β1 and α6β4 [3-4] at the cell surface and with syndecans, α-dystroglycan and 67-kDa receptor [5]. Laminin-5, one of the >15 laminin isoforms so far identified, plays an important role in cell migration during tumor invasion and tissue remodelling [1-6]. Laminin-5 also stimulates human tumor cells to form marked lamellipodia, which enhances cell migration and invasion *in vitro* [5]. The biological activity of laminin 5 is regulated by the proteolytic processing of the 3 polypeptide chains [6].

Several studies have shown a link between laminin 5γ2 expression and invasive activity of carcinoma cells from different tissues [6-9]. In vitro studies demonstrated that human colonic cancer HT29 cell lines produce and deposit laminin-5 [1]. It has been suggested that the high expression of laminin γ2 chain in invasive colon carcinoma cells is due to synergistic activation of the LAMC2 gene [3], which encodes the laminin γ2 chain, by convergence of HGF (hepatocyte growth factor) and WNT (wingless-type murine mammary virus integration-site family member) signalling pathways [3].

In previous investigations one of us (CL) found that that the cytoplasmic expression of laminin 5γ2 was present at the invading front of colonic carcinomas, supporting the view that the γ2 chain of laminin 5 could be a marker for invasiveness in colonic tumors [7, 9]. It should be understood, however, that the sequence of events pertinent to the local progression of colonic carcinomas remains unclear. In an

attempt to unveil these mechanisms, different parameters occurring at the invading edge of colonic tumors have been studied, such as expansive vs. infiltrative growth and tumor differentiation [10], presence of buds (foci with up to 4 tumor cells) [11], or of peritumoral lymphocytes (to estimate the immunological reaction of the host [12]. Some authors claim that the kinetic ability of cancer cells to migrate into the surrounding matrix (i.e. tumour cell locomotion) is the single most important parameter accountable for the local progression of tumors [13-15]. Other groups of researchers focused their interest in different tumour biomarkers such as Ki67 [16-18] and laminin-5 expression [19], p53 [20], and K-ras [21], mutations, angiogenesis [22], telomerase activation [23], and increased membrane matrix metalloproteinase (MMP) [24, 25], to name a few. Notwithstanding, despite overwhelming research, the series of events pertinent to local tumor progression have remained elusive.

Earlier studies on the tumor progression of colonic and rectal carcinomas revealed, at the invading edge, the presence of dilated neoplastic glands, some lacking a group of consecutive lining tumor cells [26-30]. These cellular gaps were called glandular pores (GPs). By challenging sections with cytokeratin MNF 116 immunostain, the structure of these glandular gaps was enhanced [27].

The phenomenon of glandular pore formation at the invading tumor front was also demonstrated in experimentally-induced colonic carcinomas in rats [31], and in Barrett´s adenocarcinomas in the human esophagus [32]. The purpose of the present work was to explore a possible association between pore formation in neoplastic glands at the growing edge of colonic carcinomas and laminin 5γ2 expression.

Material and Methods

Sections from 86 consecutive surgically resected colonic carcinomas, seen between 1993 and 1996, were investigated. Consecutive sections from the tumor were stained with hematoxylin and eosin (H&E) and with laminin 5γ2, respectively.

The preparation and characterization of laminin 5γ2 were done according to the method described elsewhere [7, 9]. Briefly, the polyclonal antibody was raised in rabbit against a fusion protein containing the C-terminus of the laminin 5γ2 chain containing the amino acid residues # 1017-1178 and GST.

Representative blocks of formalin-fixed and paraffin-embedded sections from colonic carcinomas were cut at five μm and treated with standard horseradish peroxidase avidin-biotin-complex (ABC). In brief, the sections were deparaffinized with xylene, rehydrated and microwave treated for 10 minutes at 500W in 10 mM sodium citrate buffer (pH 6). After a brief rinse in Tris buffered saline (TBS), pH 7.6, the sections were treated with 0.5% hydrogen peroxidase in distilled water for 30 minutes to block endogenous peroxidase activity, followed by 1% bovine serum albumin (BSA) in TBS for 20 minutes to prevent unspecific staining. After incubation over night at 4°C with the rabbit polyclonal antibody to the γ2 chain of laminin-5 (1:500), a biotinylated antirabbit IgG (1:200) was applied for 30 minutes. After rinsing in TBS, the biotinylated secondary antibody and horseradish peroxidase-conjugated antibiotin antibody (Vector Elite standard kit, cat. PK-6100) were applied to the sections according to the manufacturer's instructions. Peroxidase activity was visualized by applying diaminobenzidine tertrahydrochloride, 0.6 mg/mL with 0.03% H_2O_2, for 6 min. The slides were then slightly counterstained with hematoxylin, dehydrated and mounted. Parallel incubations to adjacent sections were performed in which the laminin 5γ2 chain antibody was replaced with BSA to serve as negative controls.

Laminin 5γ2 expression was assessed in five consecutive neoplastic glands with or without the glandular pores (GPs) present at the invading edge of the tumor (**Figure 1**).

Neoplastic cells seen at the free ends of the GPs expressing a stronger laminin 5γ2 immunostain than the neoplastic cells in the rest of the gland were regarded as overexpressing laminin 5γ2 (**Figure 2**). The frequency of glands overexpressing laminin 5γ2 was assessed in 100 consecutive neoplastic glands from 10 randomly selected carcinomas: 50 each with or without GPs.

Statistical analysis was performed using the chi-square test and $p<0.05$ was considered to be statistically significant.

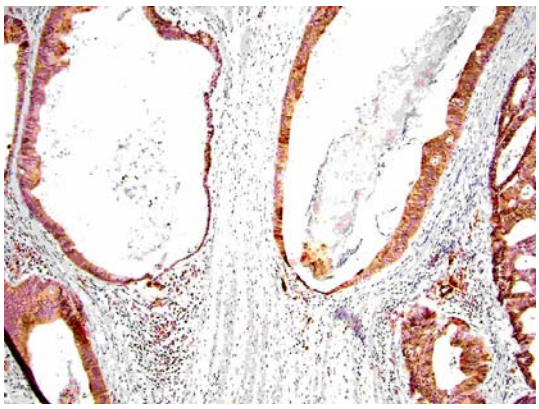

Figure 1 Entire neoplastic glands without glandular pore formation at the invading edge of a colonic adenocarcinoma expressing laminin 5γ2 (laminin 5γ2 immunostain, 20x).

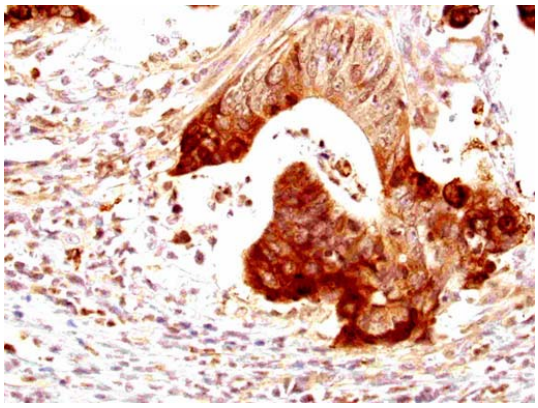

Figure 2 Neoplastic gland with glandular pore formation at the invading edge of a colonic adenocarcinoma. Note overexpression of laminin 5γ2 in the neoplastic cells at the free end of the pore (laminin 5γ2 immunostain, 40x).

Results

Frequency of Neoplastic Glands with GPs at the Invading Tumor Edge

Among the 86 colonic cancers investigated, 85% (73/86) had neoplastic glands with GPs. Of the 73 tumors with neoplastic glands with GPs, 83% (71/73) had ≥5 GPs at the invading tumor front. Neoplastic glands without pores (i.e., entire intact glands) were also present at the invading tumor front in all 73 tumors. The remaining 15% (13) were undifferentiated or signet ring-cell carcinomas.

Laminin 5γ2 Expression in Neoplastic Glands with GPs at the Invading Tumor Edge

Among the 365 neoplastic glands with GPs studied, 335 (92%) expressed laminin 5γ2. In contrast, only 17% or 63 of the 365 entire intact neoplastic glands without GPs recorded in the same cases expressed laminin 5γ2 ($p<0.05$).

Degree of Tumor penetration (TNM Classification) and the Frequency of Neoplastic Glands with GPs

Table 1 shows that 14 tumors were classified as T1-2 N0 M0 tumours, 44 as T3-4 N0 M0 tumors, 19 as T1-4 N1-2 M0 tumors and 9 as T1-4 N0-2 M1 tumors.

As summarized in **Table 1**, 95% (55/58) of the tumors without lymph node or distal metastases (N0/M0) had GPs at the growing tumor edge, whereas only 64% (18/28) of the tumors having lymph node or distal metastases (N1or M1), had GPs at the growing tumor edge ($p<0.05$).

Degree of Tumor Penetration (TNM classification) and Laminin 5γ2 Expression in Neoplastic Glands with GPs

Table 2 shows that laminin 5γ2 was expressed in 75% (249/335) of the neoplastic glands with GPs in cases without lymph node or distal metastases (N0/M0) but only in 25% (86/335) of the neoplastic glands with GPs in tumors with lymph node or distal metastases (N1/M1) ($p<0.05$).

Table 2 Degree of tumor penetration and frequency of laminin 5γ2 expression

TNM Classification	Laminin 5γ2 expression in neoplastic glands with GPs
T1-2 N0 M0	59 (18%)
T3-4 N0 M0	190 (57%)
T1-4 N1-2 M0	52 (15%)
T1-4 N0-2 M1	34 (10%)
Total	335 (100%)

Table 1 Degree of tumor penetration (TMN classification) and frequency of GP formation

TNM classification	No of tumors	Tumors with more than 5 neoplastic glands with GPs	Tumors with less than 5 neoplastic glands with GPs	Tumors with neoplastic glands without GPs
T1-2 N0 M0	14	13	0	1
T3-4 N0 M0	44	41	1 (3)	2
T1-4 N1-2 M0	19	11	0	8
T1-4 N0-2 M1	9	6	1 (4)	2
Total	86	71	2 (7)	13

The number in parenthesis indicates tumors with ≤4 neoplastic glands with GPs.

Laminin 5γ2 Overexpression in the Neoplastic Cells at the Free Ends of the GPs

Laminin 5γ2 was overexpressed at the free ends of the pores in 88% (44/50) of the neoplastic glands with GPs in 10 unselected cases (**Figure 2**). In contrast, only 14% (7/50) of the entire neoplastic glands without GPs present had occasional tumor cells overexpressing laminin 5γ2 ($p<0.05$).

Discussion

The results showed that 85% of the 86 colorectal carcinomas investigated had cellular gaps called glandular pores (GPs) at the invading tumor edge. These findings are in concert with previous investigations showing pore-formation at the invading tumor front in 81% of the colon carcinomas and in 86 % of the rectal carcinomas [28].

At the growing edge of the tumor, it was also found that 82 % of the neoplastic glands had GPs whereas only 7% of the neoplastic glands within the tumour mass showed GPs [28]. Similar to results reported by Shinto et al [34], we also found that laminin 5γ2 is more frequently expressed at the periphery of the tumor than in the tumor core.

Of the neoplastic glands with GPs investigated at the tumor front, we found that 92% expressed laminin 5γ2, thus substantiating previous studies in colonic carcinomas [7, 9]. These findings support the view that the γ2 chain of laminin 5 could be a marker for invasiveness in colonic tumors [1, 3, 5].

The expression of laminin 5γ2 was further investigated in neoplastic glands having GPs. The results indicated that laminin 5γ2 was overexpressed in the neoplastic glands with GPs than in those without pores, suggesting increased production and deposition of this macromolecule in the apical tumor cells of the GPs.

The frequency of GPs at the growing edge in tumors without lymph node or distal metastases (N0/M0) was significantly higher than in those with lymph node or distal metastases (N1 or M1). These findings are in accordance with previous studies showing that the frequency of colorectal carcinomas with GPs at the growing edge was significantly higher than in those with lymph node or distal metastases (N1 or M1) [27]. The results of the present work with laminin 5γ2 suggest that the phenomenon of neoplastic pore formation at the invading tumor edge of colonic carcinomas is associated with local tumor progression rather than the metastasizing capacity of the tumor. In this context, it should be mentioned that laminin 5γ2 expression was found to increase progressively from tubular adenomas (12%) to villous adenomas (25%) and to carcinomas (70%) [7].

We have recently found that the accumulated intra-glandular material present in the dilated neoplastic glands at the invading edge of colorectal carcinomas was rich in proteolytic enzymes [26-32, 34]. This material was subsequently discharged through the GPs into the peritumoral extracellular matrix (ECM), resulting in the breakdown of the juxtaposed ECM. It was entertained that to remodel the defective glands, malignant cells proliferating from the tip of the free borders of the pores invaded the enzymatically-disrupted matrix, aiming to achieve glandular continuity. The subsequent sealing of the glandular flaws permitted the re-accumulation of new intra-glandular proteolytic material, a mechanism

that would replicate a new wave of host invasion at the growing edge, thus ensuring a stepwise but everlasting tumour progression in untreated patients [34]. This theory is further supported in this work not only by the finding of a high frequency of GPs expressing laminin 5γ2, but also by the increased production or overexpression of laminin 5γ2 in the tumor cells located at the apical ends of the GPs. The possibility that these laminin 5γ2-overexpressing tumor cells were "organizing" the ultimate invasion of the enzymatically-digested ECM cannot not be totally excluded.

In conclusion, in agreement with previous studies in humans [26-30, 34] and in experimentally-induced colorectal carcinomas in rats [31], a high frequency of neoplastic glands with GPs were found at the advancing tumor edge of colonic carcinomas. Laminin 5γ2 expression correlated with the frequency of neoplastic glands showing GPs. The fact that laminin 5γ2 expression in GPs was significantly more frequent in tumors without lymph node and distal metastasis than in those without those variables, and that production of laminin 5γ2 was increased in the tumour cells at the apex of the glandular pores, suggest a close interaction between this adhesion-migration macromolecule, GP formation and the local progression of colonic carcinomas.

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