

Basic Study

Integrin $\alpha v \beta 6$ sustains and promotes tumor invasive growth in colon cancer progression

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Abstract

AIM: To detect the mechanism by which colon tumor escapes the growth constraints imposed on normal cells by cell crowding and dense pericellular matrices.

METHODS: An immunohistochemical study of integrin $\alpha v \beta 6$ and matrix metalloproteinase-9 (MMP-9) was performed on tissue microarrays of 200 spots, including 100 cases of colon tumors.

RESULTS: High immunoreactivity for $\alpha v \beta 6$ (73.7%; 28/38) and MMP-9 (76.5%; 52/68) was observed in invasive tumor portions. Furthermore, the effects of integrin $\alpha v \beta 6$ on tumor invasive growth in nude mice were detected. Tumor invasive growth and high expression of both $\alpha v \beta 6$ and MMP-9 were only seen in tumors resulting from WiDr cells expressing $\alpha v \beta 6$ in the tumorigenicity assay. Flow cytometry was applied to analyze $\alpha v \beta 6$ expression in colon cancer WiDr and SW480 cells. The effects of cell density on $\alpha v \beta 6$ expression and MMP-9 secretion were also detected by Biotrak MMP-9 activity assay and gelatin zymography assay. High cell density evidently enhanced $\alpha v \beta 6$ expression and promoted MMP-9 secretion compared with low density.

CONCLUSION: Integrin $\alpha v \beta 6$ sustains and promotes tumor invasive growth in tumor progression *via* a self-perpetuating mechanism. Integrin $\alpha v \beta 6$ -mediated MMP-9 secretion facilitates pericellular matrix degradation at high cell density, which provides the basis of invasive growth.

Key words: Colonic neoplasms; Integrin $\alpha v \beta 6$; Matrix metalloproteinase-9; Invasive growth

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Core tip: This study is designed to identify the mechanisms by which integrin $\alpha v \beta 6$ sustains and promotes tumor invasive growth in colon cancer progression. Our results suggested that integrin $\alpha v \beta 6$ sustains and promotes tumor invasive growth in tumor progression *via* a self-perpetuating mechanism. Integrin $\alpha v \beta 6$ -mediated matrix metalloproteinase-9 secretion facilitates pericellular matrix degradation at high cell density, which provides the basis of invasive growth.

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INTRODUCTION

Invasive growth is one of the main features that distinguish malignant tumor cells from normal cells. The mechanisms by which tumor cells escape the growth constraints imposed on normal cells by cell crowding and dense pericellular circumstance are controversial. The reason why colon cancer cells sustain invasive growth *via* a self-perpetuating manner in tumor progression is also unclear. There is a general consensus, nevertheless, that this demonstrates a cell-surface problem, and the cell adhesion molecules integrin $\alpha v \beta 6$ and matrix metalloproteinase-9 (MMP-9) are likely to be involved in tumor progression^[1,2].

Within the integrin αv subfamily, integrin $\alpha v \beta 6$ is expressed only on abnormal epithelial cells. It is highly expressed during morphogenesis and tumorigenesis^[2-4], and *de novo* expression has been observed at the margins of advanced colon tumors. One potential mechanism for the growth-promoting effect of integrin $\alpha v \beta 6$ may be *via* enhanced MMP-9 activity. The invasive growth of colon cancer cells is also likely to reflect the ability of tumor cells to digest their surrounding matrix scaffold through the secretion of MMP-9 because integrin $\alpha v \beta 6$ expression in colon cancer cells has been shown by our group to induce MMP-9 secretion^[5], and the inhibition of MMP-9 activity abolishes the integrin $\alpha v \beta 6$ -mediated growth effect^[6].

As an adhesion protein involved in both the nuclear Wnt/beta-catenin pathway and the mesenchymal transition of colorectal cancer cells, nuclear beta-catenin expression increases from the central area towards the invasive margin. It has been reported that the expression of integrin $\alpha v \beta 6$, which is also an adhesion protein, is induced during the epithelial-transition of aggressive colon carcinoma^[3,7-9]. MMP-9 overexpression related to tumor invasive growth

in gastric carcinoma has also been reported. The induction of MMP-9 mRNA in endothelial cells has been reported to be dependent on direct cell adhesion with cancer cells^[10]. The maximal expression of MMPs has also been displayed at the invasive margin of colon tumor cell islands. This finding is consistent with the observation that integrin $\alpha v \beta 6$ preferentially localizes at the leading edge of epithelial ovarian cancer with a malignant potential of invasiveness and metastasis. The consequence of integrin $\alpha v \beta 6$ -mediated MMP-9 secretion may provide the basis for a self-perpetuating system of tumor invasive growth that operates through integrin $\alpha v \beta 6$. However, the effects of both integrin $\alpha v \beta 6$ and MMP-9 on invasive growth in colon cancer progression remain controversial.

This study was designed to identify the mechanisms by which integrin $\alpha v \beta 6$ sustains and promotes tumor invasive growth in colon cancer progression.

MATERIALS AND METHODS

Cell lines and culture conditions

The human colon cancer cell lines WiDr and SW480 and the normal human keratinocyte cell line HaCaT were obtained from the ATCC (Rockville, MD, United States). SW480 cells, which lack constitutive integrin $\alpha v \beta 6$ expression, were stably transfected with pcDNA1neo constructs that contained either the $\beta 6$ gene construct or the expression plasmid only (SW480 $\beta 6$ or SW480 mock) as previously described^[11].

For flow cytometry analysis, low-density cultures were established by seeding 5 to 7.5×10^5 cells in 2.5 mL of standard medium into 6 cm-diameter tissue culture dishes or 25 cm² tissue culture flasks. High density cultures were established using identical cell numbers and medium volume seeded into 24 -well tissue culture plates (Falcon Becton Dickinson, Oxnard, CA). At 72 h, the low- and high-density cultures were approximately 40% and 100% confluent, respectively. Then, cells were harvested by trypsin/EDTA at 72 h for the assessment of integrin $\alpha v \beta 6$ expression. The ratio of cell number to surface area was in the range of 1.5 to 2×10^5 cells/cm² and 3.5 to 4×10^5 cells/cm² for the low- and high-density cultures, respectively.

Antibodies and reagents

The anti-integrin $\alpha v \beta 6$ mAb 2G2 was obtained from Biogen, and the specificity of the antibody has previously been reported^[12].

FITC-conjugated goat-anti-mouse IgG was obtained from Jackson (ImmunoResearch Laboratories, Inc., United States); anti-MMP9 mAbs-whole molecule (ab38898, Abcam, United Kingdom) was purchased from Jingmei Biotech (Shenzhen, China). Briefly, the conditioned medium (CM) for MMP-9 estimation was prepared by the removal of fetal bovine serum-containing medium and three washes of the adherent cells with phosphate-buffered saline prior to the addition of chemically defined serum-free medium.

Tissue microarray

From January 2007 to December 2007, 100 patients who underwent curative resection by the same surgical team for pathologically confirmed colon carcinoma at the Department of Pathology of Qilu Hospital (Shandong University, China) had their formalin-fixed, paraffin-embedded tissue blocks that contained colon carcinoma specimens selected. The specimens were constructed into tissue microarray (TMA; 200 spots each) slides under the Human Investigative Committee protocol of Shandong University by Chaoying Biochip Company, Ltd. (Xi'an, China). The maximum tumor diameter in each colon tumor specimen we selected was not less than 1.5 cm to suit the requirement of the TMA. After screening hematoxylin and eosin-stained (HE) slides for optimal tumor content, two cores, one obtained from tumor edge portions that contained the invasive margin of the lesion (generally located in the superficial part of the tumor) and the other from the central portion of the tumor (that contained none or little of the invasive margin, generally located in the deep part of the tumor), were obtained from each sample using punch cores; these punch cores measured 1.0 mm in the greatest dimension and were spaced 0.8 mm apart. The invasive front of the tumor has ample blood supply, and because the most biologically relevant portion of tumors with histologic heterogeneity is the area with the deepest invasion, tumor "budding" in this area is also enriched^[13,14]. The invasive fronts were defined as expanding or infiltrating in HE slides according to the morphological guidelines previously defined by Jass and colleagues.

Immunohistochemical analysis for both $\alpha v \beta 6$ and MMP-9

Immunohistochemistry (IHC) for integrin $\alpha v \beta 6$ was performed using the avidin-biotin complex (ABC) method. Overnight incubation occurred at 4 °C with primary antibody against integrin $\alpha v \beta 6$ (2G2, 1.67 μ g/mL, Biogen, Idec., United States). The antibody is specific for integrin $\alpha v \beta 6$ and does not recognize the αv or other αv integrins. Negative controls were treated identically but with the primary antibody omitted.

Immunohistochemical staining for MMP-9 was performed using a monoclonal antibody, clone ab38898, against MMP-9 (5 μ g/mL; Abcam, United Kingdom). The antibody recognizes murine and human MMP-9 but does not cross-react with the other MMP family members (MMP-1, MMP-2, or MMP-3). TMA sections, 5 μ m thick, were thaw-mounted onto Fisherbrand Super Frost/Plus slides. After air drying at 37 °C for 12 h and incubation for 20 min at 60 °C, the sections were deparaffinized and rehydrated. No antigen exposure procedure of any type was necessary. Staining was performed after incubation with the antibody at 4 °C for 12 h using labeled avidin-biotin. Negative controls underwent a similar staining procedure with the exclusion of the primary antibody

application.

Scoring of integrin $\alpha v \beta 6$ and MMP-9

The sections were assessed for both integrin $\alpha v \beta 6$ and MMP-9 immunoreactivity microscopically *via* positive DAB staining by three trained observers (Yang GY, Wang YQ and Guo S). The integrin $\alpha v \beta 6$ is a trans-membrane protein with immunohistochemical staining located in both the membrane and cytoplasm, whereas the immunohistochemical staining for MMP-9 was predominately located in the cytoplasm. The percentage of positive cells and staining intensity were determined by three observers with 100% agreement.

Flow cytometry

The cells were incubated with anti- $\beta 6$ mAb 10D5 (10 μ g/mL), E7P6 (10 μ g/mL) or an isotype-matched control IgG (10 μ g/mL) for 30 min. After washing, cells were stained with goat anti-mouse IgG conjugated with phycoerythrin/FITC for 30 min prior to flow cytometry analysis.

Tumor invasive growth in vivo

For the tumorigenicity assay, BALB/C female nude mice (6 wk of age purchased from the Animal Resource Center, Shandong University, China) were maintained under pathogen-free conditions and fed standard mouse chow and water *ad lib*. All mice within each group were inoculated with a single cell line. The cells used were WiDr wild-type and WiDr antisense $\beta 6$ transfectants. The mice received subcutaneous flank injections of approximately 2×10^6 viable tumor cells (cell counts and viability were assessed by counting cells stained with 0.4% trypan blue in a hemocytometer) suspended 0.1 mL of DMEM culture medium. Tumor sizes (breadth and length as measured with calipers) were recorded weekly. Six weeks following the last injection, the mice were sacrificed and visible subcutaneous tumors were excised. The formalin-fixed paraffin-embedded sections were analyzed for HE and IHC staining to inspect routinely for invasive growth.

MMP-9 activity assay

MMP-9 levels in TCM obtained from low-and high-density cultures were assayed using a commercially available kit, the MMP-9 activity assay system.

Gelatin zymography assay

Following electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min at RT to remove the SDS. The gels were subsequently incubated at 37 °C overnight in substrate buffer that contained 50 mmol/L Tris HCl and 5 mmol/L CaCl₂ (pH 8.0). The gels were stained with 0.15% Coomassie blue R250 (Bio-Rad, Hercules CA, United States) in 50% methanol and 10% glacial acetic acid for 20 min at room

Table 1 Clinicopathological characteristics for 100 patients with colon carcinoma

Characteristic	Number of patients	Percentage
Sex		
Male	54	54
Female	46	46
Age (yr)		
< 60	61	61
≥ 60	39	39
Tumor location		
Right	39	39
Transverse	10	10
Left colon	51	51
Depth ¹		
< 5 mm	72	72
≥ 5 mm	28	28
Maximum diameter of tumor (cm)		
≥ 1.0 and < 5.0	67	67
≥ 5.0	33	33
Tumor differentiation		
Well	19	19
Moderate	53	53
Poor	21	21
Unknown	7	7
Lymph node metastasis		
0	23	23
1-4	32	32
≥ 5	45	45
Venous vessel invasion		
Absent	42	42
Present	58	58
Tumor margin		
Expanding	81	81
Infiltrating	19	19

¹Depth of penetration beyond the muscularis propria.

temperature.

Statistical analysis

The continuous variables are expressed as the mean ± SD and were compared between groups using Student's *t* tests. The categorical variables were compared using χ^2 tests. Survival curves were drawn by the Kaplan-Meier method, and their comparisons were analyzed by the log-rank test. All statistical analyses were conducted using SPSS 20.0 statistical software (SPSS, Inc., Chicago, IL). Statistical significance was defined as a *P* value < 0.05.

RESULTS

IHC for both α v β 6 and MMP-9 in colon cancer cells

To examine the role of integrin α v β 6 in the invasive growth of colon carcinoma cells, the expression and distribution of integrin α v β 6 and MMP-9 in invasive tumors were evaluated by IHC in both the edge portion that contained the invasive margin of the lesion (generally located in the superficial part of the tumor) and in the non-invasive central area of the tumor (that contained little or none of the invasive margin, generally located in the deep part of the tumor). Two

Table 2 Effects of both integrin α v β 6 and matrix metalloproteinase-9 expression in 100 cases of colon carcinoma

Variable		Immunoreactivity of tumor samples (<i>n</i>)			
		Positive	Low expression	High expression	
α v β 6	Deep section of tumor	38	27	71.1%	11
	Superficial section of tumor	38	10	26.3%	28
MMP-9	Deep section of tumor	68	35	51.5%	33
	Superficial section of tumor	68	16	23.5%	52

¹ χ^2 test indicates that increased integrin α v β 6 expression rates are significantly different between the superficial section and deep section of the tumors (*P* < 0.05). There is also a significant difference for the increased MMP-9 expression rates between the superficial section and deep section of the tumors (*P* < 0.05). α v β 6: Integrin α v β 6; MMP-9: Matrix metalloproteinase-9.

hundred spot tissue microarrays (TMAs) including 100 cases of malignant colon tumors were used, and the clinicopathological characteristics are shown in Table 1. Table 2 summarizes the findings of both α v β 6 and MMP-9 expression on the TMAs.

Negative integrin α v β 6 expression is shown in Figure 1A. A sample with low integrin α v β 6 expression is indicated in the deep part of the tumor (Figure 1B), whereas high expression of integrin α v β 6 was detected in the tumor edge cells (Figure 1C). In paired serial sections, Figure 1D is a negative control. At the stage of tumor progression, tumor budding is exhibited as an enrichment in the invasive front^[15,16].

Intense up-regulation of α v β 6 expression, and particularly, preferential localization at the edges of both aggressive tumor islands and tumor budding are shown in Figure 1E, which is consistent with our recent report. Similarly, strong MMP-9 expression was also identified in paired serial sections as shown in Figure 1F. High integrin α v β 6 expression was detected in tumor invasive edge portions in 73.7% (28 cases of high expression/38 cases of positive expression) of cases; strong MMP-9 staining intensity was also identified in 76.5% (52 cases of high expression/68 cases of positive expression) of cases in the same tumor edge portions. These data indicate that both integrin α v β 6 and MMP-9 are strongly associated with invasive tumor growth.

To determine whether there is an association between either α v β 6 or MMP-9 expression in primary colon cancer and patient survival, a five-year follow-up of prognosis was performed. Corresponding Kaplan-Meier plot is shown in Figure 2. There was a significant difference between the integrin α v β 6 positive patients and the integrin α v β 6 negative patients (*P* = 0.048; *P* < 0.05). The survival estimates also exhibited a striking difference in the median survival: the integrin α v β 6 positive patients averaged 45.9 mo, whereas the

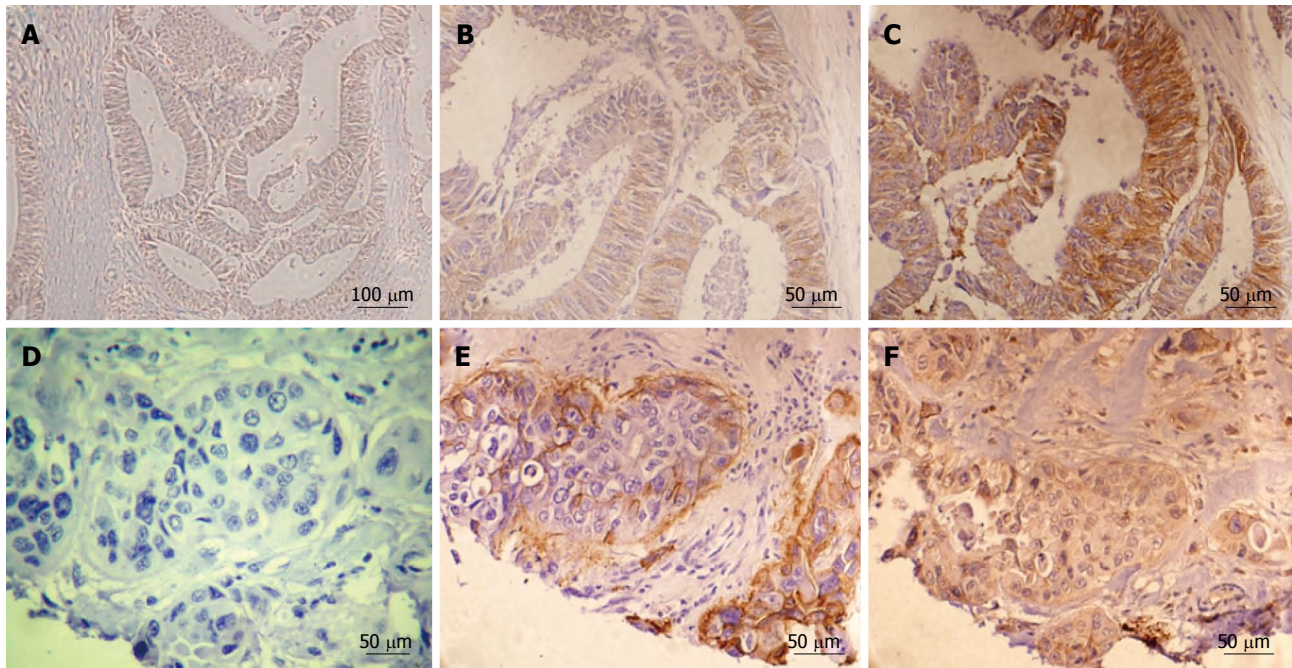


Figure 1 Representative immunohistochemistry examples for both integrin α v β 6 and matrix metalloproteinase-9 in colon cancer. Negative expression for integrin α v β 6 (A), low integrin α v β 6 expression in the tumor edge portion (B) and high integrin α v β 6 expression in the tumor central area (C). In the paired serial sections, (D) is a negative control. Positive α v β 6 expression in the edge of both invasive tumor islands and tumor budding are shown in (E). Strong matrix metalloproteinase-9 expression (F) was also identified in invasive tumor edge portions. A scale bar is shown in the right lower corner of each figure.

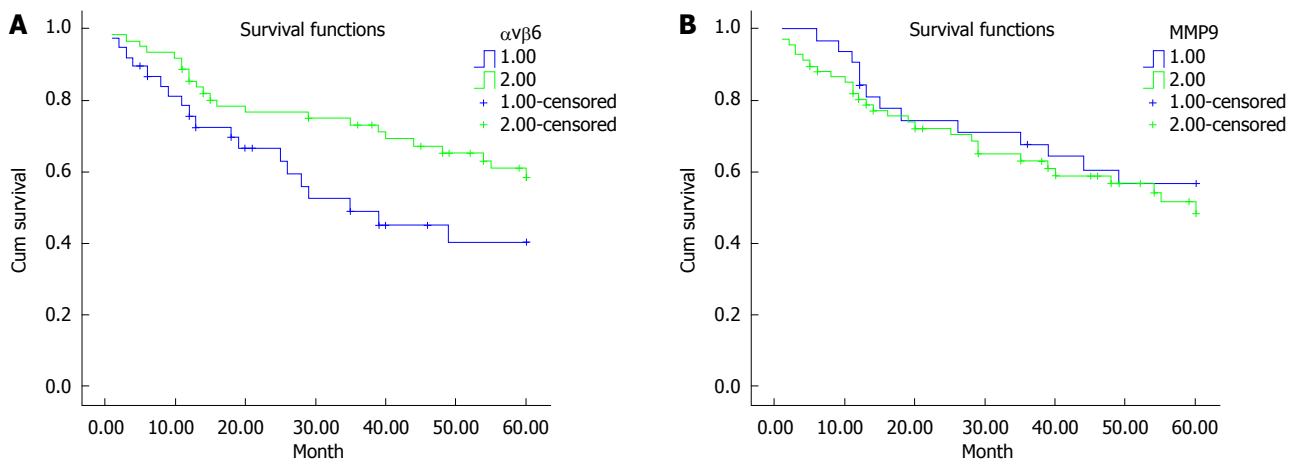


Figure 2 Kaplan-Meier survival analysis using a log-rank test. The samples were grouped according to integrin α v β 6 (A) or matrix metalloproteinase-9 (MMP-9) (B) expression level (negative or positive) and analyzed using the log-rank test for overall survival.

Table 3 Five year survival estimates for 100 patients with colon cancer

Groups	Negative/positive	n	Median survival (mo) and 95%CI	5-yr survival rate (%) and 95%CI
α v β 6	Negative	62	45.948 (40.575-51.320)	0.585 (0.452-0.718)
	Positive	38	36.114 (28.501-43.728)	0.402 (0.220-0.584) ¹
MMP-9	Negative	32	44.071 (36.812-51.330)	0.570 (0.392-0.748)
	Positive	68	41.686 (36.040-47.331)	0.485 (0.344-0.626) ²

¹Log-rank test indicates a significant survival difference between negative vs positive integrin α v β 6 expression groups ($\chi^2 = 3.919$; $P = 0.048$; $P < 0.05$);

²There was no significant difference between negative vs positive MMP-9 expression groups ($\chi^2 = 0.451$; $P = 0.502$; $P > 0.05$). α v β 6: Integrin α v β 6; MMP-9: Matrix metalloproteinase-9.

integrin α v β 6 negative patients averaged only 36.1 mo and exhibited an 18.3% lower 5-year survival as shown in Table 3. However, the log-rank test indicated that there was no significant difference in the median survival or 5-year survival between the MMP-9 positive patients and the MMP-9 negative patients. From a biological perspective, we conclude that elevated expression of integrin α v β 6 in colon cancer has been associated with a poor prognosis.

Integrin α v β 6 expression is enhanced at high cell density

To detect the mechanism by which colon carcinoma

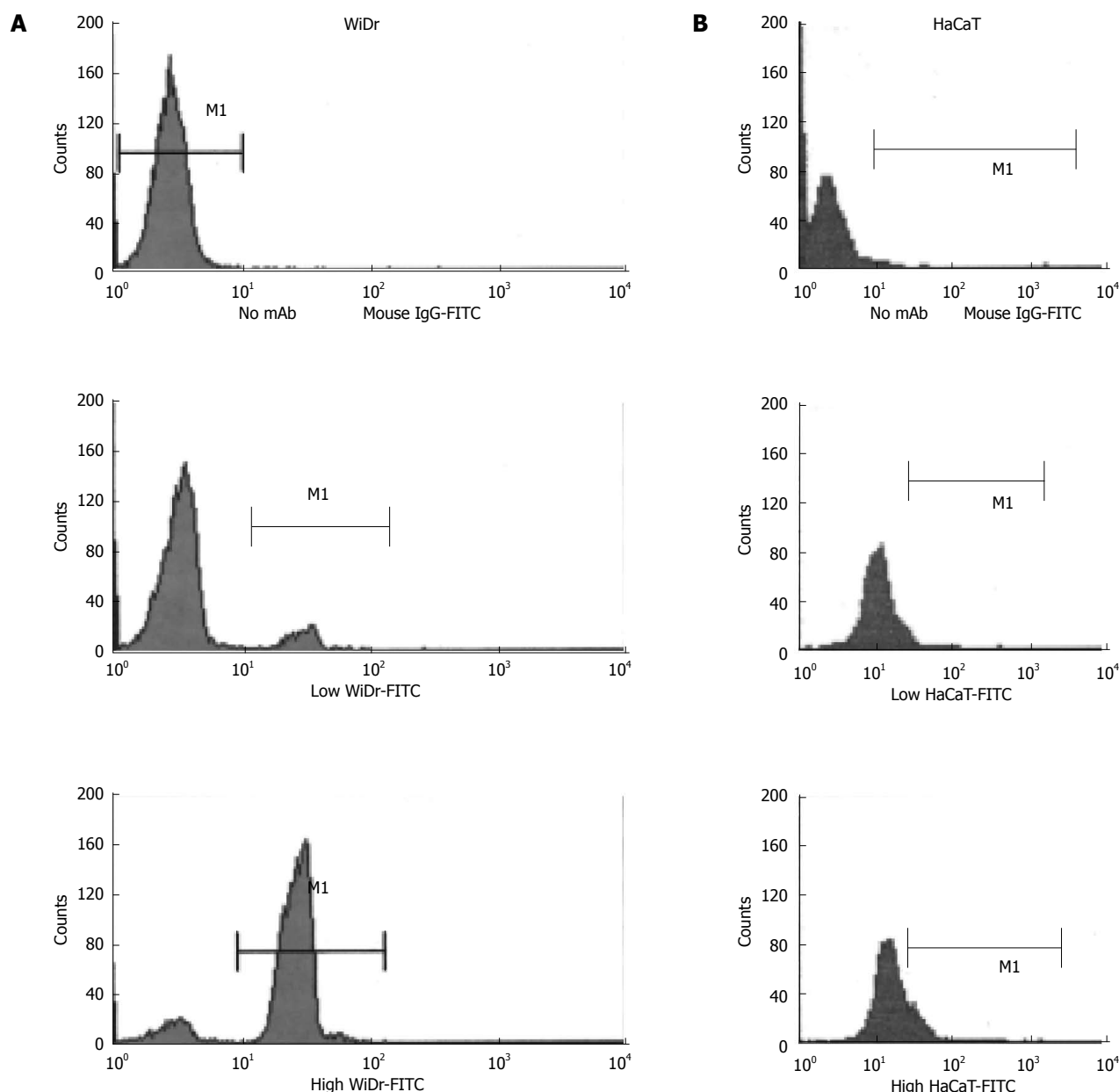


Figure 3 Effects of cell density on integrin $\alpha v \beta 6$ in both WiDr and HaCaT cells. WiDr (A) and HaCaT (B) cells harvested from low- and high-density cultures were analyzed by FACSscan for integrin $\alpha v \beta 6$ expression. The cells were incubated with either no primary antibody (upper panels) or function-blocking integrin $\alpha v \beta 6$ mAb 10D5 (lower panels) and with goat anti-mouse IgG conjugated with FITC. The data are representative of three similar experiments.

cells maintain invasive growth *via* escaping the growth constraints, the surface expression of integrin $\alpha v \beta 6$ was evaluated by flow cytometry in the human colon cancer cell line WiDr and the normal human cell line cultured at low or high cell densities. As shown in Figure 3A, the integrin $\alpha v \beta 6$ surface expression was higher in the cells with high density compared with low-density cultures, and no cell density-dependent increase in integrin $\alpha v \beta 6$ expression was displayed in HaCaT cells (Figure 3B).

Expression of integrin $\alpha v \beta 6$ enhances tumor invasive growth in nude mice

To evaluate the effect of integrin $\alpha v \beta 6$ on the invasive growth of colon cancer tumors *in vivo*, we injected

2×10^6 wild-type WiDr cells that expressed integrin $\alpha v \beta 6$ (Figure 4A) and antisense $\beta 6$ WiDr cells that expressed less integrin $\alpha v \beta 6$ (Figure 4B) into the right flank of 20 athymic mice per cell line. Tumor growth was subsequently followed for 6 wk. The tumors formed by the wild-type WiDr cells (Figure 4C) grew to a substantially larger size (breadth and length) compared with the tumors formed by the antisense $\beta 6$ WiDr cells. Of the 20 mice inoculated with antisense $\beta 6$ WiDr cells, the tumors completely disappeared in 65% (13/20) of the animals. In the remaining 7 mice, the largest tumor size was only 6 mm² (Figure 4D) compared with tumor sizes of at least 16–25 mm² in the other 20 mice inoculated with wild-type WiDr cells that expressed normal levels of integrin $\alpha v \beta 6$. These

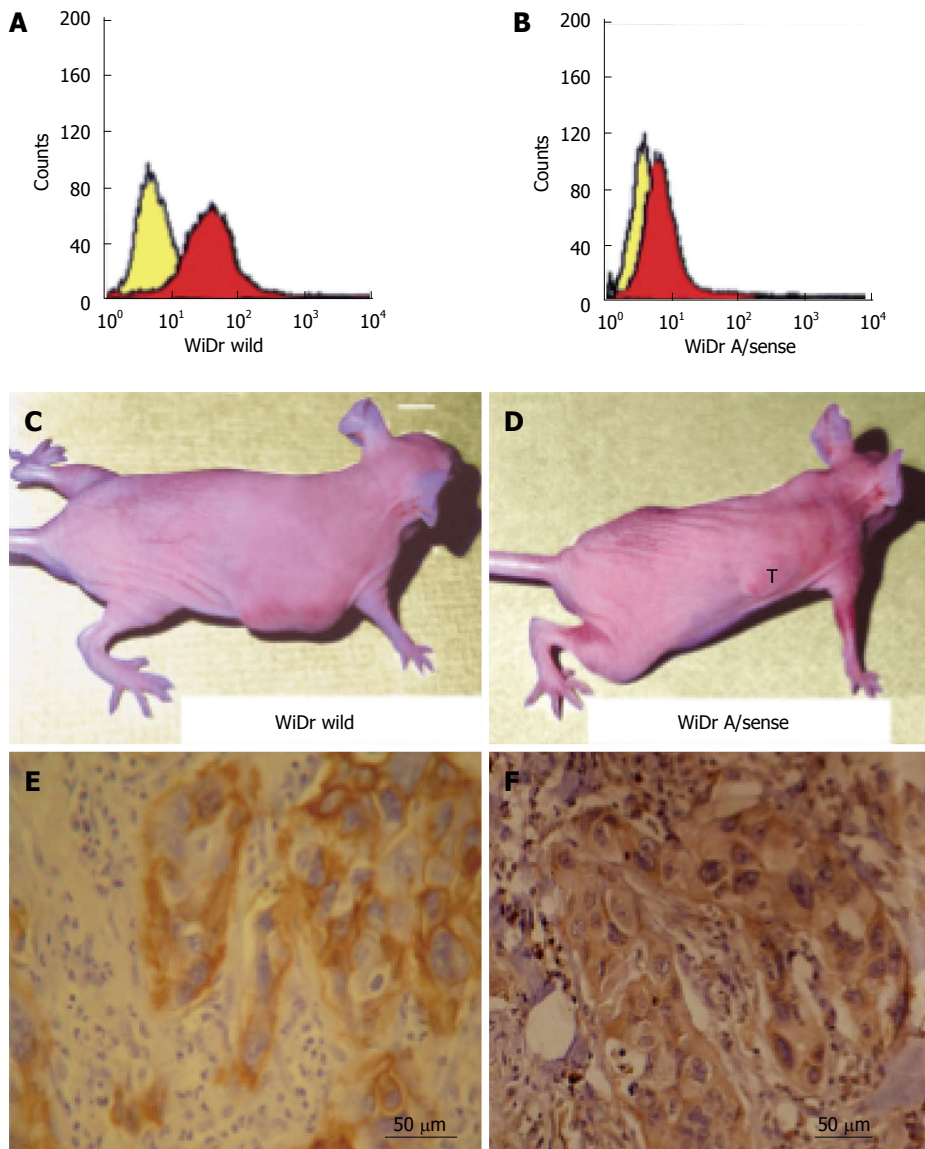


Figure 4 Integrin α v β 6 promotes invasive tumor growth in nude mice. Wild-type WiDr cells (A) and antisense β 6 WiDr transfectants (B) were analyzed by FACSscan for the expression of integrin α v β 6. The yellow and red histograms represent analyses in the absence and presence of E7P6 primary antibody; the secondary antibody (goat anti-mouse IgG) was conjugated with phycoerythrin. Tumor invasive growth after 6 wk following subcutaneous inoculation with wild-type WiDr cells (C) or antisense β 6 WiDr transfectants (D). Increased expression of integrin α v β 6 (E) and matrix metalloproteinase-9 (MMP-9) (F) was identified in serial sections of tumor xenografts inoculated with wild-type WiDr cells. One section was selected from every 10 serial sections. The figures (E) and (F) are the 1st section and 21st section selected. The thickness of each section was 5 μ m. A scale bar is shown in the right lower corner of (E) and (F).

results indicate that a fundamental difference of tumor growth exists between wild-type WiDr and antisense β 6 WiDr cells.

A significant difference in infiltrating behavior between the two tumor types was noted from routine histochemistry and IHC. Histologically, we demonstrated that the tumors formed by the antisense β 6 WiDr cells grew adjacent to, but did not infiltrate, the underlying muscle, whereas the tumors formed by the wild-type WiDr cells extensively infiltrated the underlying muscle. The results of IHC for both integrin α v β 6 and MMP-9 indicated that integrin α v β 6 was expressed highly in tumors resulting from wild-type WiDr cells as shown in Figure 4E, and MMP-9 also stained strongly in the serial sections as shown in

Figure 4F. However, in tumors resulting from antisense β 6 WiDr cells, integrin α v β 6 was barely detectable and MMP-9 was weakly expressed (data not shown). The data indicate that integrin α v β 6 expression significantly enhances invasive tumor growth *in vivo*.

MMP-9 secretion is enhanced in α v β 6-expressing cell crowding but not in normal human keratinocytes

To examine the effects of cell density on integrin α v β 6 and MMP-9 as colon carcinoma cells maintain invasive growth when cells are crowded and dense, the surface expression of integrin α v β 6 in human colon cancer cell lines was evaluated by flow cytometry as shown in Figure 5A. Wild-type WiDr and SW480 cells and β 6 SW480 cells were cultured at low and high

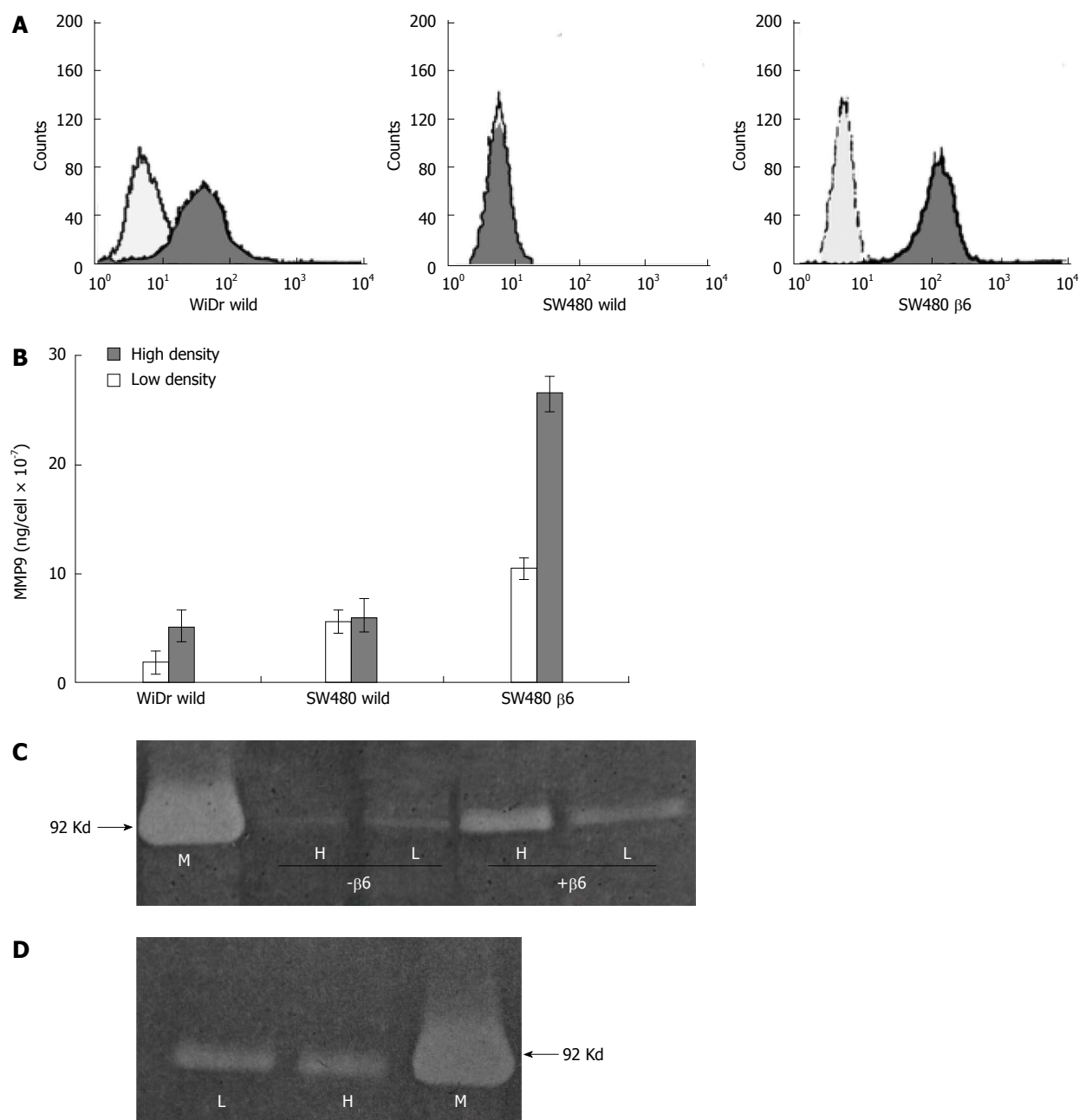


Figure 5 Effects of cell density on matrix metalloproteinase-9 secretion. A: Wild-type WiDr and SW480 cells and $\beta 6$ SW480 transfectants were analyzed by FACSscan for integrin $\alpha v\beta 6$ expression. The pale and black histograms represent analyses in the absence and presence of E7P6 primary antibody; the secondary antibody (goat anti-mouse IgG) was conjugated with phycoerythrin; B: Gelatinase B assay (Biotrak) showing levels of matrix metalloproteinase-9 (MMP-9) in TCM (20-fold concentrated). The data represent the mean MMP-9 levels per cell for three experiments; in each experiment, the TCM samples were tested in duplicate; C: Gelatin zymogram showing gelatinase activity in non-concentrated TCM from $\beta 6$ SW480 (+ $\beta 6$) and wild-type SW480 cells (- $\beta 6$) cultured at low (L) and high (H) densities. The position of purified MMP-9 (M) is shown on the left; D: Gelatin zymogram showing gelatinase activity in non-concentrated conditioned medium from HaCaT cells cultured at low (L) and high (H) densities. The position of purified MMP-9 (M) is shown on the right.

cell densities under serum-free conditions in tumor-conditioned medium (TCM) and analyzed for the presence of MMP-9 using the Biotrak MMP-9 activity assay system. As shown in Figure 5B, both the wild-type WiDr and $\beta 6$ SW480 cells secreted approximately 2-3 times more MMP-9 per cell at high cell density compared with low-density conditions. However, no increase in MMP-9 was identified in the high-density cultures of the wild-type SW480 cells relative to the low-density cultures.

To examine the effect of cell density on MMP-9 secretion in the absence of integrin $\alpha v\beta 6$, TCM from SW480 cells was analyzed for the presence of the enzyme using gelatin zymography. As shown in Figure 5C, a marked increase in the amount of MMP-9 in TCM from high-density compared with low-density cultures was identified only for the $\beta 6$ SW480 cells, which express integrin $\alpha v\beta 6$. However, no increase in MMP-9 was identified for the high-density cultures of the wild-type SW480 cells, which lack integrin $\alpha v\beta 6$. These

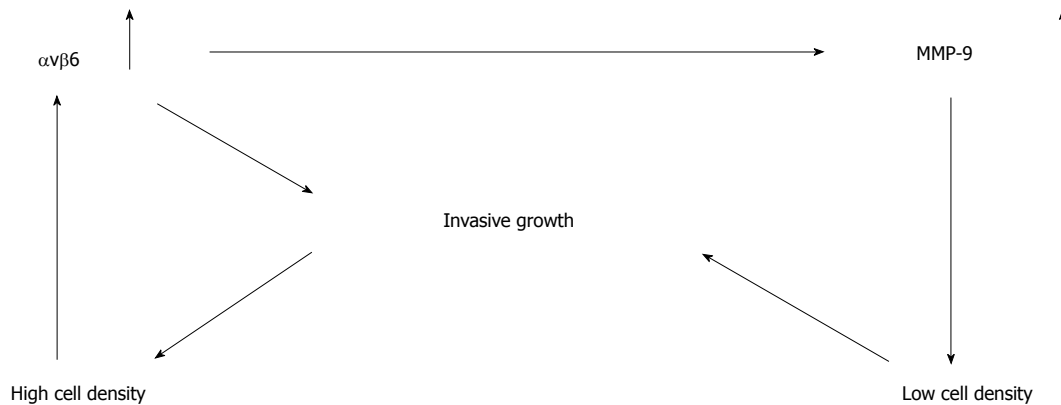


Figure 6 Model of tumor cell infiltrating growth in which integrin $\alpha v \beta 6$ provides a self-perpetuating system in colon cancer progression. $\alpha v \beta 6$: Integrin $\alpha v \beta 6$; MMP-9: Matrix metalloproteinase-9.

data indicate that the promotion of MMP-9 secretion as colon cancer cells reach confluence occurs in an $\alpha v \beta 6$ -dependent manner.

Integrin $\alpha v \beta 6$ is undetectable on HaCaT cells *in situ*, but it significantly increases in wound healing and culture-established keratinocytes^[1]. The HaCaT cells were cultured under identical conditions to the colon cancer cell lines. However, no difference in integrin $\alpha v \beta 6$ expression was identified in the HaCaT cells cultured at low vs high cell densities (Figure 3B). MMP-9 is the predominant type of collagenase in dermal keratinocytes, and in contrast to the colon cancer cell lines, MMP-9 secretion was not enhanced for the HaCaT cells cultured at high density (Figure 5D).

DISCUSSION

Invasive growth is the main characteristic feature that distinguishes malignant tumor cells from normal cells, and this relies on the capability of tumor cells to digest surrounding matrix through the secretion of MMP-9. For example, MMP activators have been implicated in colon cancer invasive growth^[17]. The increased expression of integrin $\alpha v \beta 6$ has also been demonstrated to promote cell growth^[17-19] and to induce MMP-9 secretion both *in vitro* and *in vivo*^[5,6,20]. Furthermore, the inhibition of MMP-9 activity abolishes the integrin $\alpha v \beta 6$ -mediated growth effect. Nevertheless, the effect of integrin $\alpha v \beta 6$ on invasive growth remains unknown. According to Weiss *et al.*^[21], the term "crowdedness" by its mere literal sense signals that the size and conformation of a test particle dictates if it feels an environment is crowded. Here, cell crowding and density are references to a specific environment in which the cell concentration or density is more crowded than the normal status. The expression of certain genes that regulate cell growth in colon cancer may be cell density-dependent. Takeha also reported that increased MMP-9 expression in primary cancer cells is associated with infiltrating growth in human colorectal cancers, essentially distributed

along the invasive margin. We have demonstrated that increased high integrin $\alpha v \beta 6$ immunoreactivity is coincident with high MMP-9 staining in invasive tumor portions that contain the invasive margin of the lesion compared with the non-invasive tumor central area (that contains little or none of the invasive margin). Furthermore, the culmination of our observations is detected in the invasive front (at the stage of tumor development when tumor cells migrate into and invade the surrounding tissue either as single cells or in collective clusters, thereby forming an invasive front), especially in the crowded cell zone, of colon cancer tumors. In this study, we demonstrate that the colon cancer cell lines WiDr wild-type and SW480 $\beta 6$ transfectants compared with HaCaT normal human keratinocyte cells at high cell density exhibit evidently enhanced integrin $\alpha v \beta 6$ expression. The present study suggests that integrin $\alpha v \beta 6$ is preferentially expressed over normal human cells during invasive growth when colon cancer cells become crowded and dense in progression. These data also indicate that invasive tumor growth is strongly associated with the induced expression of integrin $\alpha v \beta 6$ at a high cell density.

The maximal expression of MMP-9 has been identified at the invasive margin of tumor cell islands in colon cancer^[12,14,20]. We have also reported that integrin $\alpha v \beta 6$ induces MMP-9 secretion^[12,17]. In contrast, no density-dependent secretion of MMP-9 was identified for colon cancer cells that lacked integrin $\alpha v \beta 6$. We also recently reported that the down-regulation of constitutive integrin $\alpha v \beta 6$ expression dramatically reduced MMP-9 levels in the cultures of human colon cancer cell lines^[22]. Therefore, the present study suggests that integrin $\alpha v \beta 6$ -mediated MMP-9 secretion at high cell density provides the basis for a self-perpetuating system of invasive tumor growth for integrin $\alpha v \beta 6$ -expressing cells that operates through integrin $\alpha v \beta 6$.

In vivo, a variety of factors and events affect tumor cell invasive growth. To associate cell density-dependent increase in integrin $\alpha v \beta 6$ expression and MMP-9 secretion with invasive growth *in vivo*,

we performed tumorigenesis assays. At least in theory, some requirements, such as the need for angiogenesis, degradation of ECM, and local invasive growth, can be recapitulated in this system. Our experimental data demonstrated that tumor invasive growth was enhanced for the wild-type WiDr cells that expressed normal integrin $\alpha v \beta 6$ compared with the antisense $\beta 6$ WiDr cells. More importantly, the tumors formed from wild-type WiDr cells infiltrated deep into the underlying muscle, whereas the tumors from WiDr antisense $\beta 6$ cells grew adjacent to, but did not invade, the underlying muscle; the increased expression of integrin $\alpha v \beta 6$ and MMP-9 was only identified in the tumors resulting from wild-type WiDr cells. Furthermore, according to the five-year follow-up data for 100 cases of patients with colon cancer, the log-rank test indicated that there was no significant survival difference between the MMP-9 positive patients and the MMP-9 negative patients ($P = 0.48$). There was, however, a significant difference between the integrin $\alpha v \beta 6$ negative patients and the integrin $\alpha v \beta 6$ positive patients ($P = 0.048$). Biologically, we observed that the elevated expression of integrin $\alpha v \beta 6$ in colon cancer was associated with a poor prognosis. This finding provided strong evidence for integrin $\alpha v \beta 6$ as a potential independent factor of invasive growth.

We suggest that the expression of integrin $\alpha v \beta 6$ in colon cancer cells as they become crowded and dense during progression may be critical to the invasive growth that is characteristic of this type of tumor. We propose a self-perpetuating model that explains tumor cell invasive growth as shown in Figure 6. The stimulatory effect of cell density on integrin $\alpha v \beta 6$ expression is enhanced in colon cancer cells that express integrin $\alpha v \beta 6$. As a consequence of this integrin $\alpha v \beta 6$ -mediated MMP-9 secretion, pericellular matrix degradation is facilitated, which helps to overcome cell crowding. This effect reduces the matrix density and facilitates invasive growth because a high collagen density exerts an inhibitory effect on colon cancer invasive growth *in vitro*, and the cells are converted from low to high density again. The repeating cycle from low to high cell densities resembles a closed infinity symbol. Our study indicated that suppression of integrin $\alpha v \beta 6$ expression inhibits both tumor invasive growth and high secretion of MMP-9 in tumor xenografts in nude mice.

Integrin $\alpha v \beta 6$ is expressed in basal keratinocytes during wound healing and in culture-established keratinocytes^[23,24]. Our study indicated that no increase in integrin $\alpha v \beta 6$ expression or MMP-9 secretion was identified at a high cell density. Invasive growth contributes to the embrace of novel therapeutic strategies that target specific cancer cell characteristics.

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COMMENTS

Background

To detect the mechanism for tumor invasive growth in colon cancer progression, an immunohistochemical study of integrin $\alpha v \beta 6$ and matrix metalloproteinase-9 (MMP-9) was performed on tissue microarrays of 200 spots, including 100 cases of colon tumors.

Research frontiers

The results showed that high immunoreactivity for integrin $\alpha v \beta 6$ (73.7%; 28/38) and MMP-9 (76.5%; 52/68) was observed in invasive tumor portions. Furthermore, the effects of integrin $\alpha v \beta 6$ on tumor invasive growth in nude mice were detected. Tumor invasive growth and high expression of both integrin $\alpha v \beta 6$ and MMP-9 were only seen in tumors resulting from WiDr cells expressing integrin $\alpha v \beta 6$ in the tumorigenicity assay. Flow cytometry was applied to analyze integrin $\alpha v \beta 6$ expression in colon cancer cell lines WiDr and SW480. The effects of cell density on integrin $\alpha v \beta 6$ expression and MMP-9 secretion were also detected by Biotrak MMP-9 activity assay and gelatin zymography assay. The study indicated that high cell density evidently enhanced integrin $\alpha v \beta 6$ expression and promoted MMP-9 secretion compared with low density.

Innovations and breakthroughs

Integrin $\alpha v \beta 6$ sustains and promotes tumor invasive growth in tumor progression via a self-perpetuating mechanism. Integrin $\alpha v \beta 6$ -mediated MMP-9 secretion facilitates pericellular matrix degradation at high cell density, which provides the basis of invasive growth.

Peer-review

This is an interesting manuscript. In this manuscript, the mechanisms by which integrin $\alpha v \beta 6$ sustains and promotes tumor invasive growth in colon cancer progression are identified. The study design is good, and the results are interesting.

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