

Altered Calreticulin expression in human colon cancer: Maintenance of Calreticulin expression is associated with mucinous differentiation

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Abstract. Calreticulin is an endoplasmic reticulum luminal calcium-binding chaperone involved in various cellular functions and is a ligand for the scavenger receptor CD91. Recent studies, based on proteomic approaches on whole tissue samples containing both neoplastic and non-neoplastic cells, have shown alterations of Calreticulin expression in colon carcinomas, albeit with divergent results. The aims of this study were: 1) to assess the expression of Calreticulin and its receptor CD91 in 58 human colon adenocarcinomas, compared with paired normal mucosa, using a semi-quantitative immunohistochemical analysis, and 2) to examine associations between the tumour phenotypic features, and Calreticulin and/or CD91 expressions. Calreticulin expression was down-regulated in 51.7% human colon adenocarcinomas. Accordingly, quantitative immunoblot analysis showed that Calreticulin expression was significantly lower in human colonic cancer cell lines than in preparations of isolated human normal colonic epithelial cells. CD91 was co-expressed with Calreticulin in both normal colonic epithelial cells and pericryptic myofibroblasts. Calreticulin and CD91, that characterize the 'amateur phagocyte' function of epithelial cells, were both down-regulated in 48% of adenocarcinomas. Finally, Calreticulin expression was significantly associated with the mucinous differentiation of the tumour. Collectively, these results show that Calreticulin is likely to play a pivotal role in the differentiation of human colonic adenocarcinomas.

Introduction

Calreticulin, an ancient and highly conserved protein, is an ER luminal calcium-binding chaperone involved in many cellular functions such as regulation of calcium homeostasis and

calcium-dependent pathways (1,2), lectin-like chaperone activity (3), modulation of cell adhesion (4-6), and modulation of steroid-sensitive gene expression (7). In addition, Calreticulin appears to play an important role in the immune system, since it is involved in the assembly of major histocompatibility complex class I proteins, necessary for class I antigen presentation (8-11). Calreticulin also plays a role in the removal of apoptotic bodies and in the immunomodulation via its receptor CD91, known as an endocytic scavenger receptor expressed by antigen-presenting cells (12-17).

A proteomic study in mice, based on the comparison of colonic epithelial cells from normal and MIN mice (i.e. mice with an APC gene mutation developing numerous intestinal adenomas), shows a down-regulation of Calreticulin expression in MIN adenomas (18). Two other studies, comparing the expression of Calreticulin in human colon adenocarcinomas to that of normal epithelium by proteomic analysis on whole tissue samples containing both neoplastic and non-neoplastic cells, show divergent results i.e. an up-regulation (19) or a down-regulation (20) of Calreticulin in human colon adenocarcinomas. Collectively, these findings suggest that Calreticulin regulation is an early event and could participate in colon carcinogenesis. However, up to now, the comparative expression of Calreticulin in neoplastic and normal paired colonic epithelial cells by immunohistochemistry has not been addressed. This is an important point knowing that Calreticulin may be found not only in epithelial cells but also in the tumour stromal reaction.

The aims of this study were: 1) to assess by immunohistochemistry Calreticulin expression in a series of 58 human colon adenocarcinomas compared with paired normal mucosa, 2) to compare Calreticulin expression in colon adenocarcinomas with that of its receptor CD91, and 3) to examine possible associations between the tumour phenotypic features i.e. tumour localisation, TNM staging, histological grading, presence of a mucinous differentiation, presence of a lymphoid stromal reaction, and Calreticulin and/or CD91 expressions.

Materials and methods

Tumor samples. Fifty-eight consecutive patients with primary adenocarcinoma treated at the Department of Surgery (Centre Hospitalier Universitaire of Nantes) were included in the study.

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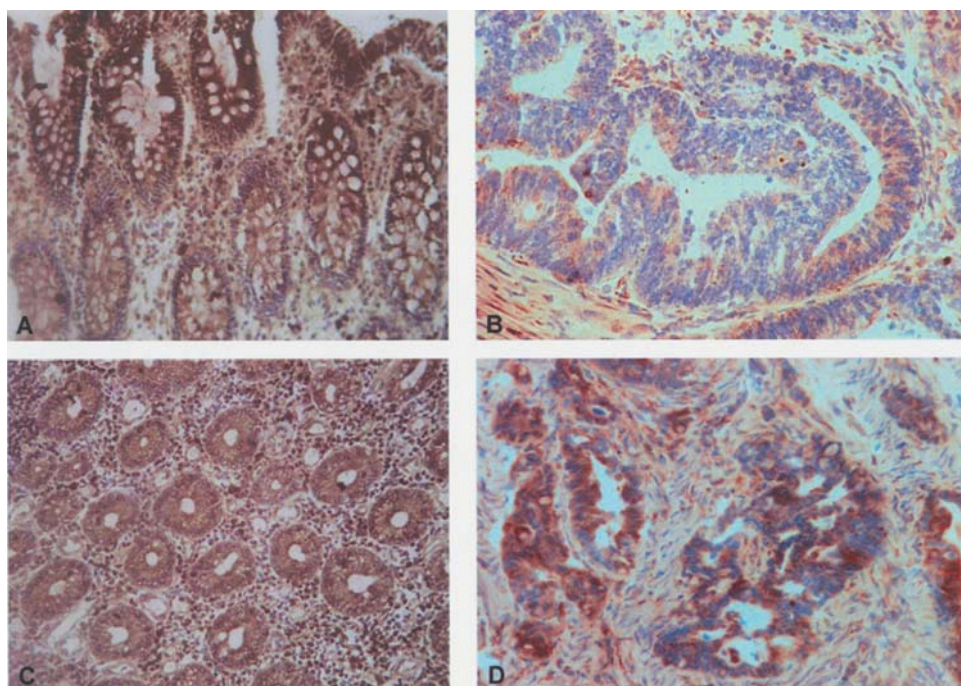


Figure 1. Calreticulin immunohistochemistry in human colonic adenocarcinomas compared with normal paired epithelium. Frozen sections of human adenocarcinomas and normal paired epithelium were stained with the polyclonal anti-Calreticulin antibody and counterstained with hematoxylin. In normal colonic mucosa, Calreticulin was expressed by colonic epithelial cells all along the crypt and also by lamina propria cells (A and C). In colonic adenocarcinomas, Calreticulin expression in neoplastic cells was either down-regulated (B) or similar (D) to that of normal paired epithelium. Calreticulin was also expressed in the stroma of colon carcinoma. Original magnification x200.

There were 38 men and 20 women with a median age of 72.5 years (mean 70.4; range 35-87). Patients condition was assessed according to the system of staging primary tumour/regional lymph nodes/distant metastasis (TNM) described in the AJCC Cancer Manual. The World Health Organisation Classification of tumours was used to determine histological classification. The 58 patients were classified into the TNM stages as follows: stage 1, 2 patients; stage 2, 33 patients; stage 3, 14 patients; and stage 4, 9 patients. Thirty-six tumors originated in the right colon, 22 in the left colon. None of the patients underwent radiotherapy or chemotherapy before surgery. The tumours, histologically classified according to the WHO classification, were divided into 44 'classic' adenocarcinomas and 14 mucinous adenocarcinomas. 'Classic' adenocarcinomas are usually gland-forming and classified as well, moderately or poorly differentiated adenocarcinomas. They display no mucinous differentiation. Mucinous adenocarcinomas are characterised by pools of extracellular mucins that contain malignant epithelium as acinar structures, strips of cells or single cells, and representing >50% of the lesion. Finally, the lymphoid stromal reaction within the tumour was graded as 0, +, ++.


For the 58 patients studied, snap frozen tumor samples and paired normal colonic tissue taken at a 10 cm distance from the tumour, were available, collected by the Biobank of Institut Régional du Cancer Nantes Atlantique, according to the guidelines of the French Ethics Committee for Research on human tissues.

Immunohistochemical studies. An immunoperoxidase technique was performed by using a streptavidin-biotin

peroxidase method on acetone-fixed frozen sections, according to the manufacturer's instructions (LSAB kit, Dako Cytomation, Trappes, France). The primary antibodies used were Calreticulin (1:800; Stressgen, Ann Arbor, MI, USA) and CD91 (1:300; Dako Cytomation). The chromogen used was DAB (3,3'-diaminobenzidine tetrahydrochloride) and tissue sections were counterstained with hematoxylin. Appropriate negative controls (omission of the primary antibody) were used throughout.

An immunolocalisation of Calreticulin and CD91 expression was performed, as well as a semi-quantitative evaluation comparing the expression of tumour cells with that of the paired normal colonic epithelium. An assessment of the number of positive cells in the tumour, as well as the intensity of the cytoplasmic staining was performed. When at least 50% of tumour cells showed a very low staining or scored negative, the tumour was considered as down-regulated for Calreticulin or CD91 expression relative to the paired normal epithelium.

For co-expression studies, immunofluorescence followed by confocal microscopy was performed on 4 cases of colon cancer and paired normal epithelium. The following double-staining combinations were performed on 8- μ m para-formaldehyde-fixed frozen sections: Calreticulin (rabbit, 1:800)/CD91 (mouse, 1:200); α -smooth muscle actin (α -SMA, rabbit, 1:400, Abcam, Oxon, UK)/CD91 (mouse, 1:200). After 1 h of incubation with the primary antibodies, sections were incubated with a mixture of alexa fluor 488-conjugated goat anti-mouse antibody and alexa fluor 568-conjugated goat anti-rabbit antibodies (1:200, Molecular Probes, Eugene, OR, USA). Nuclear staining was performed with TOPRO-3 (1 μ M,

 SPANDIDOS PUBLICATIONS Probes). Imaging was performed on a Leica TCS-SP laser scanning microscope (Leica, Heidelberg, Germany) as previously described (21). Quantification of the overlay of labeling was carried out using the MetaMorph software, as previously described (22).

Isolation of human normal colonic epithelial cells. Human colonic epithelial cells were isolated from histologically normal colon taken at a 10 cm distance from the tumor of surgical resections for colon cancer using a non-enzymatic dissociation technique as described previously (23). Preparations of colonocytes were devoid of contamination by immune cells (23).

Human colonic cell lines. The following human colonic cancer cell lines were used: the undifferentiated HT29 cell line (24) and its differentiated clonal derivatives HT29-Cl.16E, HT29-Cl.19A (25), as well as SW1116, SW480, SW620 (26) and Colo 320 (27). These cell lines were cultured in DMEM (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and used at post-confluency or until full differentiation.

Immunoblot analysis. For total protein extraction, human normal colonocytes as well as the various cell lines were lysed in RIPA buffer supplemented with protease inhibitors, and centrifuged. Proteins (5 μ g) were separated by electrophoresis on 10% polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and transferred onto polyvinylidene difluoride membranes (PVDF, Invitrogen). After blocking, membranes were incubated with a mixture of rabbit polyclonal antibodies against Calreticulin (1:20,000, StressGen) and mouse monoclonal antibodies against β -actin (1:10,000, Sigma, St. Louis, MO, USA), and then with a mixture of alkaline phosphatase-conjugated anti-rabbit (1:10,000; Amersham Biosciences, Piscataway, NJ, USA) and anti-mouse antibodies (1:2000, Sigma). Immunoreactive proteins were detected with a fluorescence scanner (Storm, Amersham) using ECF substrate according to the manufacturer's instructions (Amersham). Quantification was performed using the Image Quant software (Amersham), and results were expressed as the ratio of Calreticulin/ β -actin.

Statistical analyses. Statistical analyses were performed with GraphPad Prism version 4.0 (GraphPad software Inc., San Diego, CA, USA). Mann-Whitney U test was used to compare Calreticulin/ β -actin levels between cancer cell lines and preparations of normal colonic epithelial cells.

Fisher's exact method was used to test the associations between CD91 and/or Calreticulin expressions and one of the following phenotypic features of the tumours: localisation (right or left colon), TNM staging, presence of a mucinous component, presence of a lymphoid stromal reaction. A two-tailed probability of 0.05 was accepted as statistically significant.

Results

Down-regulation of Calreticulin expression in human colonic adenocarcinomas compared with paired normal epithelium.

In normal colonic mucosa, Calreticulin was expressed by colonic epithelial cells all along the crypt and also by lamina propria mononuclear cells, endothelial cells, fibroblasts and muscularis mucosa smooth muscle cells. All these cells displayed a strong cytoplasmic staining. Epithelial cells also displayed a membrane staining (Fig. 1A and C).

In 58 cases of adenocarcinoma, Calreticulin immunostaining was compared with paired normal colonic mucosa at distance from the tumour using a semi-quantitative evaluation. Calreticulin was found to be down-regulated in 30 out of the 58 cases (51.7%) (Fig. 1B). In the majority of the down-regulated cases, nearly all tumour cells scored negative or very weakly positive. In a few tumours considered as down-regulated, the expression of Calreticulin was more heterogeneous: the majority of tumour lobules scored negative, but a few lobules, representing 10-30% of tumour cells, displayed a Calreticulin expression similar to that of the normal paired epithelium. In the other 28 cases (48.3%), the intensity of Calreticulin staining was homogeneous and similar to that of the normal paired epithelium (Fig. 1D). In most cases, the stromal reaction within the tumour, i.e. fibroblasts, endothelial cells, inflammatory cells, smooth muscle cells, strongly expressed Calreticulin.

Down-regulation of Calreticulin expression in colonic cancer cell lines compared with normal isolated colonic epithelial cells. To fully explore Calreticulin expression in human normal isolated colonic epithelial cells and in several human colonic cancer cell lines, quantitative immunoblots using anti-Calreticulin and anti- β -actin antibodies were performed in triplicate on four preparations of normal colonic epithelial cells and seven colonic cancer cell lines. Calreticulin antibody detected a strong unique band at ~60 kDa (Fig. 2A). Fig. 2A, which illustrates a representative immunoblot, shows that Calreticulin expression was higher in isolated colonic epithelial cells than in the various cancer cell lines tested. Quantification of Calreticulin and β -actin was performed on several immunoblots using the Image Quant software for each sample (Fig. 2B). A significant down-regulation of Calreticulin/ β -actin ratio (60%) was observed in the seven colonic cancer cell lines tested (0.67 ± 0.05), compared with the four isolated colonic epithelial cell preparations (1.14 ± 0.05 ; $p=0.0001$).

Down-regulation of CD91 expression in human colonic adenocarcinoma compared with paired normal epithelium

CD91 expression in human normal colonic mucosa. As Calreticulin is a binding partner of CD91, we evaluated the expression of CD91 in 25 out of the 58 cases previously studied with Calreticulin. In normal colonic mucosa, CD91 was always expressed by epithelial cells (Fig. 3A and C). The staining was mainly cytoplasmic. CD91 was also expressed by pericryptic cells, likely myofibroblasts, and by lamina propria mononuclear cells (Fig. 3A and C). Double immunostaining followed by confocal microscopy, using anti-CD91 and α -SMA antibodies, confirmed that α -SMA-positive pericryptic myofibroblasts expressed CD91 (Fig. 4, upper panel). Confocal microscopy also showed that epithelial cells co-expressed CD91 and Calreticulin (Fig. 4, lower panel). Quantification of the overlay of labeling with the image processing software MetaMorph showed that the majority of

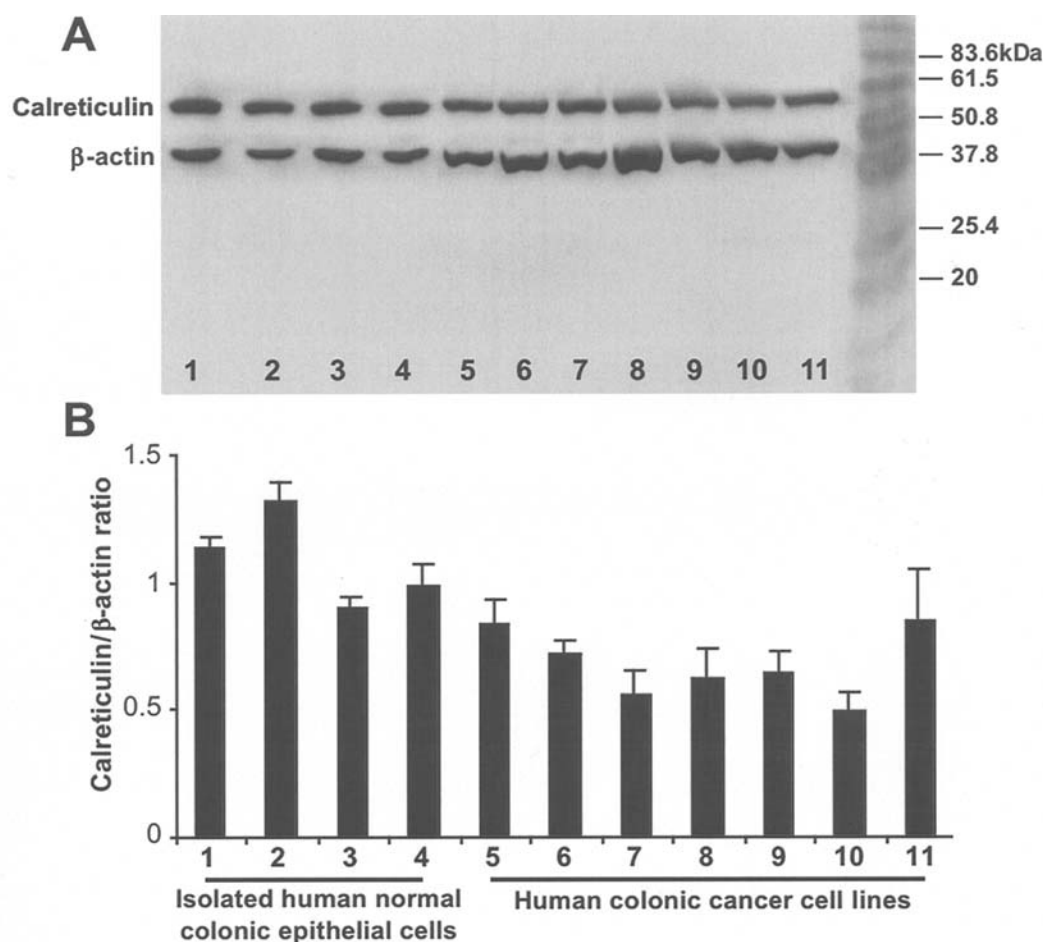


Figure 2. Immunoblot analysis of Calreticulin expression in colonic cancer cell lines compared with normal isolated colonic epithelial cells. (A) A representative immunoblot shows Calreticulin and β -actin expression in four preparations of isolated human normal colonic epithelial cells (lanes 1-4) and in seven human colonic cancer lines (lane 5, SW1116; lane 6, SW620; lane 7, SW480; lane 8, HT29; lane 9, HT29-Cl.19A; lane 10, HT29-Cl.16E; lane 11, Colo320). Molecular weight markers are shown on the right. (B) A quantitative analysis of the bands was performed with the image Quant software on three different immunoblots (mean \pm SE). A down-regulation of the Calreticulin/ β -actin ratio was observed in the seven colonic cancer cell lines tested, compared with the four preparations of isolated colonic epithelial cells.

CD91 co-localised with Calreticulin in epithelial cells ($67.5 \pm 7\%$, mean \pm SE of 10 different regions analysed).

CD91 expression in colon adenocarcinomas. In adenocarcinomas, CD91 was down-regulated in 18/25 cases (72%) compared with paired normal epithelium, with a complete loss of expression in 4 cases (Fig. 3B). In 7/25 cases (28%), CD91 expression in tumour cells was similar to that of normal epithelial cells (Fig. 3D). In most cases, the stromal reaction within the tumour was strongly labeled. Of the 25 cases, 12 (48%) showed a down-regulated expression of both CD91 and Calreticulin.

Association between pathological features of the tumours, and the expression of Calreticulin and/or CD91. In 25 cases, we examined a possible association between the pathological features of the tumours, i.e. localisation of the tumour (right or left colon), TNM staging, mucinous differentiation, presence of a lymphoid stromal reaction, and the expression of Calreticulin and/or CD91.

The only statistically significant association was found between Calreticulin expression and the mucinous differentiation of the tumour ($P=0.006$). The majority of mucinous adenocarcinomas (5/6=83%) expressed Calreticulin at a level

similar to that of paired normal epithelium, using a semi-quantitative immunohistochemical scoring method. Accordingly, among the 17 cases down-regulated for Calreticulin, 16 (94%) were devoid of mucinous component.

Moreover, when extending the analysis to the series of 58 cases immunostained with anti-Calreticulin, an association was found again between the Calreticulin expression and mucinous adenocarcinomas ($P=0.002$). The vast majority of mucinous adenocarcinomas (12/14=86%) expressed Calreticulin. Accordingly, the vast majority (90%) of tumours displaying a down-regulated expression of Calreticulin were devoid of mucinous differentiation, and are thus considered as 'classic' adenocarcinomas according to the WHO classification.

Discussion

The main findings of this study are: 1) Calreticulin expression, compared to paired normal epithelium, is down-regulated in 51.7% of human colon adenocarcinomas in a series of 58 cases; 2) the Calreticulin receptor CD91, known to be expressed by macrophages, is also expressed by normal colonic epithelial cells and pericyptic myofibroblasts; 3) both

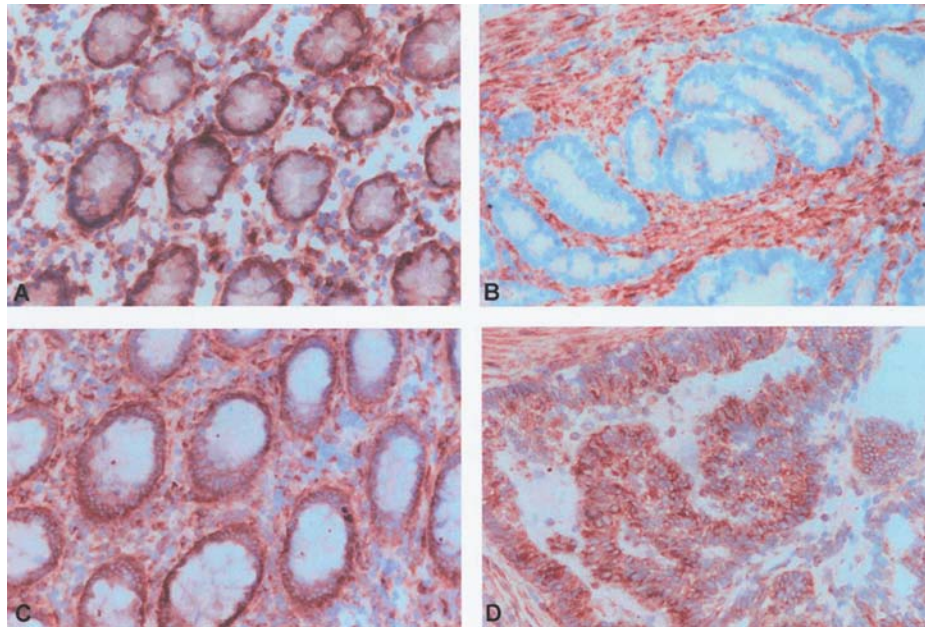


Figure 3. CD91 immunohistochemistry in human colonic adenocarcinomas compared with normal paired epithelium. Frozen sections of human adenocarcinomas and normal paired epithelium were stained with the monoclonal anti-CD91 antibody and counterstained with hematoxylin. In normal colonic mucosa (A and C), CD91 was expressed by colonic epithelial cells. CD91 was also expressed in the lamina propria by pericryptic cells, likely myofibroblasts, and by mononuclear cells. In colonic adenocarcinomas (B and D), CD91 expression in neoplastic cells was either down-regulated (B) or similar to that of normal paired epithelium (D). CD91 was also expressed in the stroma of colon carcinoma. Original magnification x200.

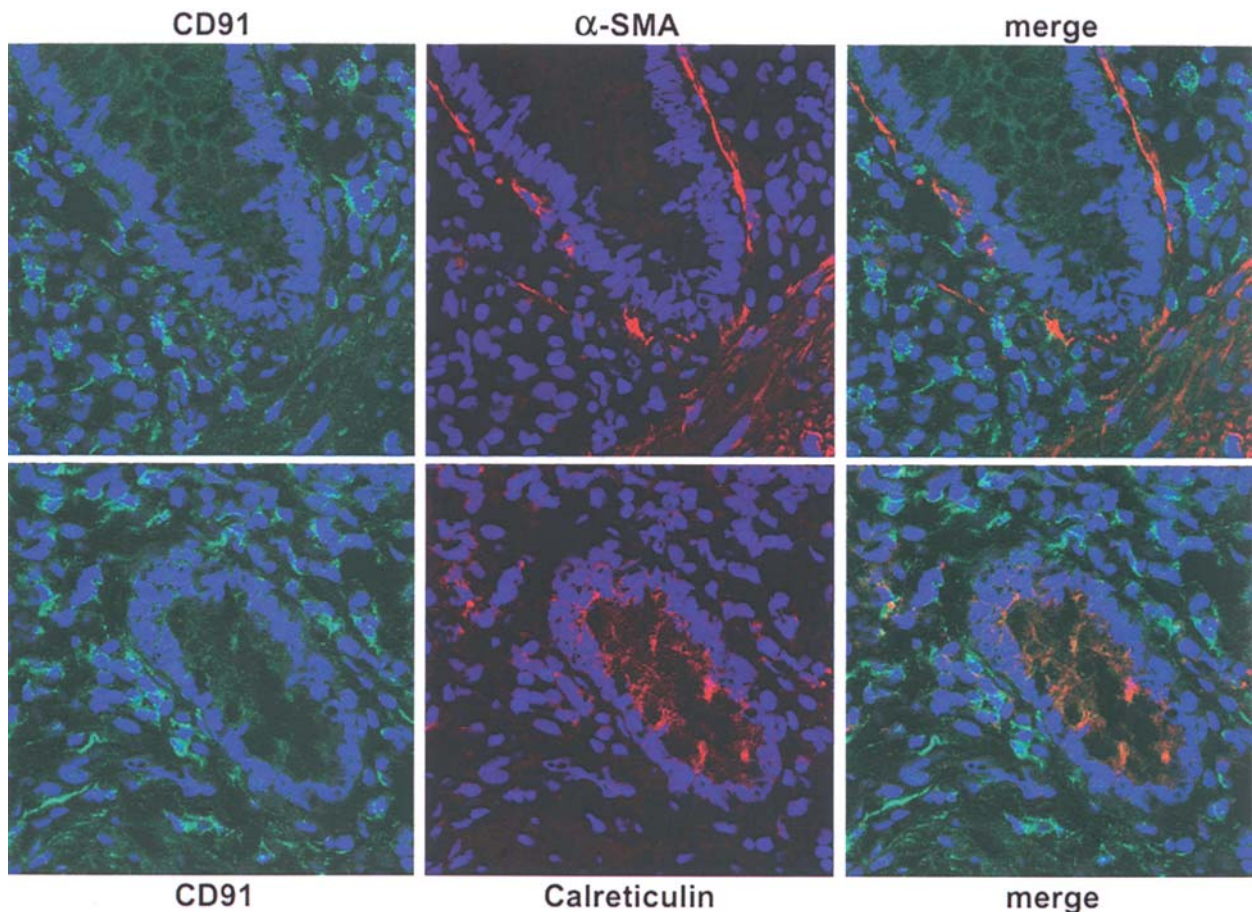


Figure 4. Co-expression of CD91 and α -SMA in human colonic myofibroblasts, and of CD91 and calreticulin in human colonic epithelial cells. Double immunostaining of CD91 (green) and α -SMA (red) or calreticulin (red) was performed on frozen sections of human normal colon and examined by confocal microscopy as described in Materials and methods. Nuclei appear in blue. The myofibroblasts surrounding colonic crypts, scoring positive for α -SMA, expressed CD91 (upper panel). Colonic epithelial cells, as well as some lamina propria immune cells, co-expressed CD91 and Calreticulin (lower panel). Original magnification x630.

CD91 and Calreticulin are down-regulated in 48% of colonic adenocarcinomas, and 4) Calreticulin expression is significantly associated with the mucinous differentiation of the tumour.

Calreticulin expression in human colorectal adenocarcinomas has been studied by proteomic analysis with divergent results reported, i.e. an up-regulation (19) or a down-regulation (20). These discrepancies may be explained by the fact that these studies were restricted to whole samples of colorectal adenocarcinomas including both neoplastic and non-neoplastic cells. As Calreticulin is expressed by several cell types, analysis based on whole sample lysates may be invaluable for assessing Calreticulin expression specifically in tumour cells. Up to now, the comparative expression of Calreticulin in neoplastic and normal colonic epithelial cells by immunohistochemistry has not been addressed. The findings of our study demonstrate a down-regulation of Calreticulin expression in 51.7% of human colorectal adenocarcinomas at the protein level using a semi-quantitative immunohistochemical analysis already validated for various proteins in colon cancer (21) or other neoplasms (28,29). Our results were confirmed by a quantitative immunoblot analysis comparing Calreticulin expression in human isolated normal colonic epithelial cells with that of human colonic cancer cell lines. A significant down-regulation of the Calreticulin/ β -actin ratio was found in the seven colonic cancer cell lines tested, compared with the four preparations of isolated colonic epithelial cells.

This study is the first to assess the expression of Calreticulin together with its binding partner CD91 in colorectal adenocarcinomas. CD91 was identified initially as a receptor for α 2-macroglobulin (30,31), and then as the receptor for several heat-shock proteins including Calreticulin (12). CD91 is present on cells of the monocytic lineage, but also in other cell types including hepatocytes, intestinal epithelial cells, and fibroblasts (32). Recent studies have shown that CD91 complexed with Calreticulin participates in driving the clearance of epithelial apoptotic cells by other epithelial cells, so-called 'amateur phagocytes', in a model of mammary gland involution and in mammary epithelial cell lines (33). These findings suggest that Calreticulin and CD91 expressed by epithelial cells can play a role in the maintenance of tissue homeostasis. In line with this study, we extended the concept of 'amateur phagocyte' to human normal colonic epithelial cells and pericryptic α -SMA-positive myofibroblasts, both co-expressing Calreticulin and CD91. This association could play a role in the autophagocytosis process/apoptotic cells removal, and contribute to colonic epithelial homeostasis.

Our study further demonstrates that CD91 expression was down-regulated in 72% of adenocarcinomas. In addition, 48% of adenocarcinomas showed a down-regulated expression of both CD91 and Calreticulin in neoplastic epithelial cells. These findings, showing a dramatic decrease in Calreticulin/CD91 expression, are in line with the concept that tissue homeostasis is altered in cancer.

Finally, these findings led us to examine the possible associations between important phenotypic features of these tumours: tumour localisation, TNM staging, histological grading, presence of a lymphoid stromal reaction, mucinous

differentiation, and the expression of Calreticulin and/or CD91. The only statistical association was found between the presence of Calreticulin and the mucinous differentiation of the tumour. Within the 58 cases studied, 14 were classified as mucinous adenocarcinomas, and 12 of these 14 cases expressed Calreticulin. Accordingly, among the cases down-regulated for Calreticulin expression, the vast majority (90%) was devoid of mucinous component. Interestingly, another study has established a molecular link between Calreticulin and mucin expression (34). Using human colonic cancer cell lines and immunoprecipitation with anti-mucin and anti-Calreticulin, McCool *et al* (34) were able to show that Calreticulin is involved in the folding of MUC2 but not of MUC5AC. As MUC2 overexpression is a feature of mucinous carcinomas of the colon (35), and was also found in our cases of mucinous carcinomas (data not shown), these findings strongly suggest that the maintenance of Calreticulin expression is essential for chaperoning MUC2 synthesis in mucinous adenocarcinomas.

In conclusion, we show herein for the first time a molecular/morphological association that is of importance for the understanding of the differentiation of human colonic adenocarcinomas.

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