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Mucosal immune environment in colonic carcinogenesis: CD80 up-regulation in colonic dysplasia in ulcerative colitis

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ABSTRACT

Background: In patients with ulcerative colitis (UC) the inconsistency between the rate of dysplasia and actual cancer incidence suggests the presence of an immunosurveillance mechanism. The aim of our study was to analyse the expression of CD80 and CD86 during the different stages of UC-associated and in non-inflammatory carcinogenesis.

Patients and methods: Sixty-two patients affected with UC, UC with colonic dysplasia, UC and cancer, colonic adenoma, or colonic cancer and 11 healthy subjects were enroled in our study. Tissue samples were taken from surgical specimens during colonic resection or during colonoscopy. Mucosal mRNA expression of CD80 and CD86 was quantified with real time polymerase chain reaction (RT-PCR). CD80, CD86 and p53 expressions and lamina propria mononuclear cell populations (CD3, CD20 and CD68) were analysed by immunohistochemistry. Mucosal levels of IL-1β, IL-2 and IFN-γ were measured with immunometric assays.

Results: Among UC patients, CD80 protein expression was higher in those with dysplasia ($p = 0.017$). In non-inflammatory carcinogenesis pathway CD80 protein and mRNA expressions were lower compared to the corresponding steps in the UC pathway. CD80 expression was directly correlated with the lamina propria mononuclear cell populations (T and B lymphocytes and monocytes). CD80 protein, but not CD80 mRNA, expression was significantly and directly correlated with IL-2 expression.

Conclusion: CD80 resulted to be up-regulated in UC with dysplasia, while it was down-regulated in cancer. CD80 mucosal levels correlate with lamina propria T-cell and with IL-2 expression suggesting that it may elicit an active role in the immunosurveillance mechanism.

1. Background

Patients affected by ulcerative colitis (UC) are at greater risk of colorectal cancer[1] with a cumulative risk at approximately 8% 20 years after the initial diagnosis and up to 18% after 30 years.[2,3] Pre-malignant histological alterations in UC patients are broadly referred to as dysplasia, rather than adenoma, since dysplasia is frequently not

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polypoid.[4,5] While Lim et al. observed that at least 25% of UC patients were diagnosed with low grade dysplasia (LGD) during a 10 year follow-up, some studies have suggested that it develops in all UC patients if the follow-up is sufficiently long.[6,7] The inconsistency between the cumulative rate of dysplasia and actual cancer incidence suggests the presence of a mechanism that can at least partially prevent malignant progression from inflammation to cancer.

Cancer immunoediting, mediated by CD8 and CD4 T cells, macrophages and natural killer (NK) cells may lead to cancer cell destruction (cancer immunosurveillance) with complete extrinsic tumour elimination resulting in definitive protection.[8] It is well known, nonetheless, that some tumour cells escape immunosurveillance leading to unrestrained neoplastic cell growth and metastatic diffusion. The immune escape mechanism is thought to be facilitated by both the mechanisms of tumour cell defence and of immune system failure.[9,10] It has recently been demonstrated that activation of tumour-specific and cytotoxic activity of CD8 T cells and the tumour-selective migration of CD4 T helper cells take place during the early stages of colorectal cancer.[11]

The actual activation of naive T cells requires the engagement of T cell receptors (TCR) with antigen-specific, major histocompatibility complex (MHC)-restricted receptors in the presence of co-stimulation molecules on the surface of antigen-presenting cells.[12–14] MHC–antigen-complex presentation in the absence of a co-stimulatory signal induces T-cell anergy.[15] Costimulatory signals can be provided by the interaction of CD80 or CD86 with CD28 (on the surface of the T cell) that induces tyrosine phosphorylation of several substrates and enhances T cell activation promoted by MHC–TCR interaction.[16–18]

Immunogenic proteins, such as the products of oncogenes or oncosuppressor mutated proteins, are potentially expressed by colorectal cancer cells, but they are not rejected by the immune system. In fact, even antigen-presenting cells infiltrating colorectal carcinomas, which express classes I and II MHC, do not express costimulatory CD80 and CD86 molecules.[19] Tirapu et al. in fact, reported dominant inhibitory effects on tumour immunity by CD80 low expression.[20] Nevertheless, CD80 expression can be induced by an oncogenic insult and CD80 expression by human carcinoma cell lines, up-regulated by IFN-γ, was attributed to the early stages of tumourigenesis when they were selected.[21,22] In fact, in a previous study, we observed a significant CD80 over-expression in UC patients with dysplasia and higher cumulative dysplasia rates in CD80 positive UC patients.[23]

The aim of our study was, then, to compare the expression of both CD80 and CD86 co-stimulatory molecules each step along the way during the different stages of carcinogenesis in UC and in non-inflammatory carcinogenesis. The secondary objective was to evaluate the mucosal expression of CD80 and CD86 in the immune response to neoplastic progression, correlating it to the lamina propria mononuclear cell populations (T and B lymphocytes and macrophages) and to the cytokine network.

2. Patients and methods

2.1. Patients

The study was performed according to the principles of the Helsinki declaration of 1983. All the subjects participating gave informed consent. Final assignment to one of the five groups was made on the basis of a postoperative histologic examination of resected colonic tissue samples obtained during colonic resection or endoscopical polipectomy. The patients were thus diagnosed as affected with: UC, UC with colonic dysplasia, UC and cancer, colonic adenoma (colonic dysplasia not in UC) or colonic cancer. A group of healthy subjects were also enroled. The UC patients were diagnosed on the basis of their clinical features, laboratory inflammation testing and endoscopic and histological findings.[24]

2.2. Study design

Tissue samples were taken from surgical specimens during colonic resection in the patients affected by dysplasia or cancer or during endoscopical polipectomy in subjects with adenomas. Two 3 mm mucosa specimens were obtained from the sigmoid region (20–25 cm from the anal verge) during a colonoscopy prescribed as a screening procedure in healthy subjects and those patients whose exams showed no evidence of any current pathology were considered controls for our study. All mucosa specimens were divided in two parts: one frozen in liquid nitrogen and then stored at −80 °C for molecular analysis and the other preserved in 10% formalin solution for histological analysis. The patients' medical records were reviewed and their demographic and clinical data, including duration and disease extension, symptoms, therapy, dates and findings with regard to colonoscopies and colonic biopsies, surgery and its indication, findings and histology, the dates of follow-up examinations and vital signs were collected.

2.3. mRNA expression of CD80 and CD86

2.3.1. RNA isolation

The total RNA was extracted from frozen colonic mucosa using acid guanidinium thiocyanate–phenol–chloroform following the Chomczynski and Sacchi method.[25] The RNA concentration was quantified spectrophotometrically, and the integrity of the sample was assessed by electrophoresis on 2% agarose gel (FMC BioProduct, Rockland, ME, USA) containing ethidium bromide. The quality of the isolated RNA was assessed using RNA 6000 Nano Assay and the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The Bioanalyzer used gel electrophoresis in the confines of a micro-fabricated chip and highly sensitive laser induced fluorescence detection using an intercalating dye added to the polymer.

2.3.2. Reverse transcription

Complementary DNA (cDNA) was synthesised using 2 μg of RNA, which was reverse transcribed in a final volume of 40 μl in the presence of 1× PCR buffer, and 1 mM each of

dNTPs (dATP, dTTP, dCTP, and dGTP), 1 U/μl RNase inhibitor, 2.5 μM random hexamers, 2.5 U/μl of Murine Leukaemia Virus (Perkin–Elmer, Foster City, CA, USA). Executed in a Perkin–Elmer GeneAmp PCR System 2400, the reverse transcription reaction was performed at 25 °C for 10 min, at 42 °C for 15 min and at 99 °C for 5 min. The cDNA was stored at –20 °C.

2.3.3. *SYBR Green I real time polymerase chain reaction (PCR)*

An ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used to develop a quantitative real time PCR with fluorescent dye SYBR Green methodology. The reaction was performed in a 96-well thin-wall optical plate. PCRs were performed in a 25 μL final volume containing 1× SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 300 nM primers (each) and 1 μL cDNA template. After a 2-min step at 50 °C to allow UDG to act and a second, 10-min long one, at 95 °C to inactivate the UDG and activate Taq polymerase, samples were subjected to 45 cycles for 45 s at 94 °C followed by 45 s at 62 °C for CD80, CD86 and GAPDH.

All the determinations were performed in triplicate in order to estimate their reproducibility. Samples in which the cDNA was omitted were used as negative controls. Each assay included 'no template' controls and a standard curve for each gene of interest. Nucleotide sequences for sense and antisense primers were synthesised to generate the following oligonucleotides: CD80 amplicone (121 bp) forward: CTCACTTCTGTTCAGGTGTTATCCA; reverse: TCCTTTTGCCAGTAGATGCGA; CD86 amplicone (81 bp) forward: CATCACAAAAAGCCCACAGGA; reverse: AGGTTGACTGAAGTTAGCAAGCACT.

2.3.4. *Quantification of gene expression*

The mRNA quantities of the unknown samples were determined on the basis of standard curves containing 108, 107, 106, 105, 104, 103, 102, 10 copies/ml for each of the primer pairs considered. The CD80 and CD86 mRNA quantities were divided by the GAPDH mRNA quantity for each sample to calculate the normalised amount of transcripts.

2.4. *Pathology assessment*

2.4.1. *Histology*

The specimens were fixated in 10% neutral buffered formalin and then dehydrated and embedded in paraffin wax. Three micrometre sections were produced and then stained with haematoxylin–eosin. A gastrointestinal pathologist (A.C.) examined the microscopic slides from the surgical specimens. The grade of histological inflammation was defined using Floren's score.[26] The Vienna classification of epithelial neoplasia of the gastrointestinal tract was adopted.[27]

2.4.2. *Immunohistochemistry*

CD80, CD86 and p53 expressions as well as lamina propria mononuclear cell (LPMC) populations were analysed by immunohistochemical staining. The p53 expression was used to mark the different steps of carcinogenesis. The CD3 expression identified the mature T cell populations, the CD20 identified the B lymphocytes and the CD68 localised the macrophages. Immunohistochemical analyses were performed using mouse monoclonal antibodies: anti CD3 (clone UCHT1, Caltag, Bayshore Burlingame, CA, USA, dilution 1:100), CD20 (clone H299 (B1) – Coulter Inc., Fullerton CA, USA, dilution 1:100), anti-CD80 (MAB-140, R&D Systems, Inc., USA, dilution 1:100), anti-CD86 (AF-141-NA, R&D Systems, Inc., USA, dilution 1:100), anti-CD68 (MAB20401, R&D Systems, Inc., USA, dilution 1:100) and anti-p53 (Novacastra, Newcastle upon Tyne, UK, dilution 1:100). Immunostaining was performed using an avidin–biotin–peroxidase conjugate and 3-3′-di-aminobenzidine tetrahydrochloride chromogen as a substrate (ABC Kit, Vector Laboratories, Burlingame, CA, USA; and DAB kit Dako, Glostrup, Denmark). The peroxidase of the detecting system reacted with 3′3′-diaminobenzidine (DAB) which was added to the slides, staining the positive cells brown. Sections were independently evaluated by a gastrointestinal histopathologist (A.C.) who graded the expression of CD80, CD86, CD3, CD20, CD68 and p53 on a semi quantitative scale (no, low, moderate or high expression). Ten random fields (×60 magnification) were examined from each sample.

2.5. *Immunoassay*

The biopsies for immunoassays were mechanically homogenised in 100 μl of phosphate buffer pH (7.4). The total protein content was measured following Bradford's method[28], using Bio-Rad Laboratory kits (Hercules, California, USA). The homogenates were then diluted to a final volume of 500 μl with 0.9% NaCl saline containing 4% BSA. Mucosal levels of IL-1β, IL-2 and IFN-γ were measured with immunometric assays (Immulite analyzer; Diagnostics Products Corporation DPC, Los Angeles, California, USA). The sensitivity of the assays was 1.5 pg/mL (IL-1β), 2 pg/mL (IL-2) and 1.7 pg/mL (IFN-γ). Mucosal levels of cytokines refer to the protein concentration.

2.6. *Statistical analyses*

Since no assumption on the data distribution normality was possible, they were presented as median (interquartile range) unless otherwise specified. Non-parametric Kruskal–Wallis ANOVA and two tailed Mann–Whitney *U*-test with Bonferroni correction were used, when appropriate, to compare relative levels of CD80 and CD86 according to dichotomous variables. The linear associations between the relative levels of CD80 and CD86 and the local, systemic, and inflammation parameters as well as the clinical, endoscopic and histological disease activity measures were quantified using Kendall's τ correlation test. Frequency analysis was performed using Fisher's exact test. Statistical significance was set at $p < 0.05$ for all tests.

3. Results

3.1. *Patients characteristics*

Thirty-six consecutive UC patients who underwent proctocolectomy or the completion of proctocolectomy procedure in our department were enroled in this study. Thirty-seven consecutive subjects were enroled to analyse the non-inflamma-

tory carcinogenesis pathway. The patients' characteristics are outlined in Table 1.

3.2. *Co-stimulatory molecules expression in the UC and in the non-inflammatory carcinogenesis pathways*

CD80 and CD86 expressions along the inflammatory and non-inflammatory carcinogenesis pathways are shown in Fig. 1 and their significant correlations in Table 2. Among UC patients, CD80 protein expression was significantly higher in those with dysplasia. Higher, but not statistically significant, levels of CD80 mRNA were also observed in these patients. This difference was not present in patients with non-inflammatory colonic carcinomas in whom CD80 protein and mRNA expressions were lower compared to the corresponding steps in the UC pathway. CD80 protein was expressed mainly by LPMCs but also by epithelial cells. CD80 mRNA expression was directly correlated with CD80 protein and CD86 mRNA expression levels. It was also directly correlated with the LPMCs and there was a marked correlation with histological and clinical inflammatory severity. CD80 protein expression was significantly and directly correlated with all three LPMC populations, with histological inflammatory severity and with UC extension. CD80 protein, but not CD80 mRNA, expression was significantly and directly correlated with IL-2 expression.

In UC patients the CD86 protein expression was significantly higher than that in the corresponding steps of non-inflammatory carcinogenesis; CD86 mRNA expression was higher in patients with UC and UC and dysplasia than healthy subjects and in patients with adenoma, respectively. They were, nonetheless, similarly expressed in each pathway in the three steps of carcinogenesis. An inverse correlation between CD86 mRNA and carcinogenesis progression was observed. CD86 mRNA expression was also directly correlated with lamina propria monocytes (CD68+). There was a marked correlation, likewise, with histological and clinical inflammatory severity. CD86 protein, but not CD86 mRNA, expression was significantly correlated with IFN-γ expression.

Table 1 – Patients' characteristics.

Carcinogenesis in UC	Ulcerative colitis (UC)		UC and dysplasia		UC and cancer	
Subjects		19		10		7
Median age (IQ range) years		51 (45–60)		55 (42–57)		54 (49–64)
Gender (male/female)		11 versus 8		6 versus 4		4 versus 3
Disease duration (IQ range) years		12 (2–15)		13 (7–15)		12 (7–18)
Histology	No dysplasia	15	HGD	2	T1 N0 M0	5
	Previous dysplasia	4	Low grade dysplasia (LGD)	8	T2 N0 M0	1
					T3 N1 M0	1
Procedures	RPC	14	RPC	9	RPC	6
	Proctocolectomy	1	Transanal resection	1	proctectomy	1
	Subtotal colectomy	1				
	Proctectomy	3				
Non inflammatory carcinogenesis	Healthy subjects		Adenoma and dysplasia		Cancer	
Subjects		11		11		15
Median age (IQ range) years		69 (61–73)		61 (53–67)		64 (61–74)
Gender (male/female)		7 versus 4		6 versus 5		7 versus 8
Histology	Normal	11	HGD (4 FAP)	6	T1 N0 M0	2
			LGD	5	T2 N0 M0	4
					T3 N0 M0	6
					T3 N1 M0	2
					T3 N2 M1	1
Procedures	Colonoscopy	11	RPC	3	Right colon resection	6
			Subtotal colectomy	1	Left colonic resection	2
			Right colonic resection	2	Anterior resection	5
			Transverse colonic resection	1	Transanal resection	2
			Endoscopic polypectomy	3		

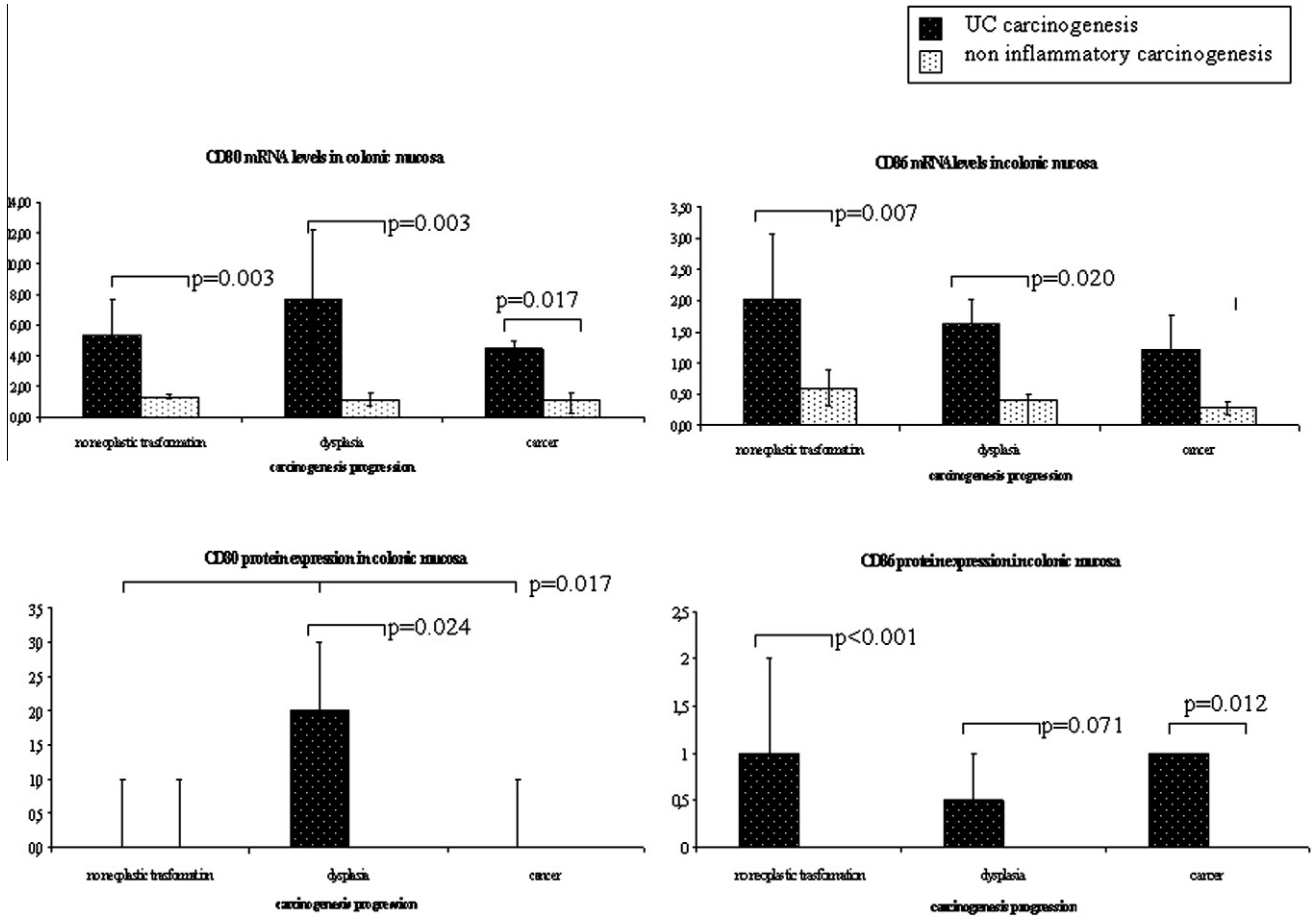


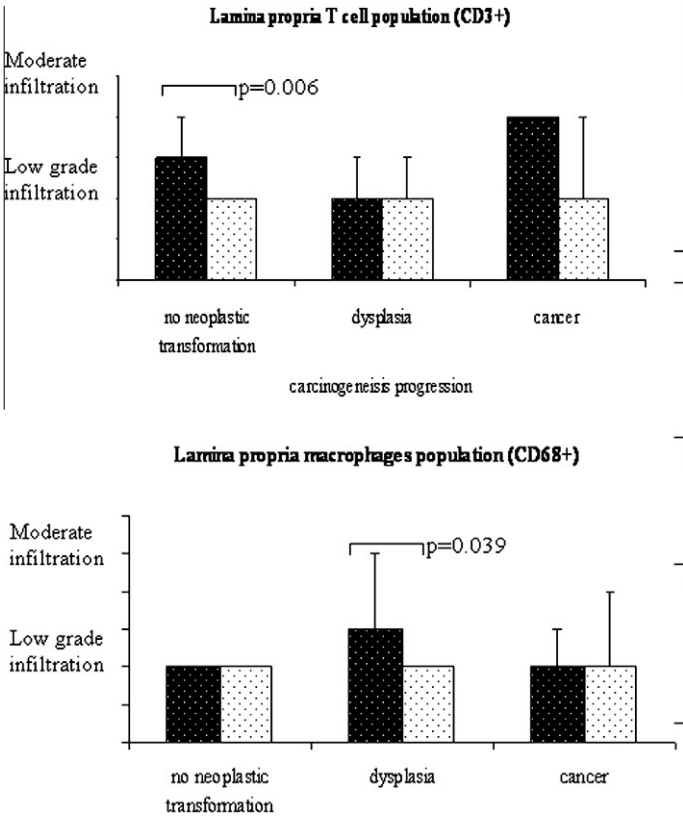
Fig. 1 – CD80 and CD86 expressions in the colonic mucosa at the different steps of carcinogenesis.

Table 2 – Significant correlation between co-stimulatory molecules and carcinogenesis and inflammatory response.

		Kendall's τ	p-Level
CD80 mRNA	CD80 protein expression	0.26	0.018
	CD86 mRNA	0.40	0.000
	CD3+ population	0.30	0.020
	CD20+ population	0.28	0.027
	CD68+ population	0.30	0.019
	Histological inflammation severity	0.60	0.000
CD80 protein expression	CD80 mRNA	0.26	0.018
	CD3+ population	0.21	0.027
	CD20+ population	0.31	0.001
	CD68+ population	0.24	0.012
	IL2	0.21	0.034
	Histological inflammation severity	0.31	0.001
	UC extension	0.33	0.025
CD86 mRNA	CD80 mRNA	0.40	0.000
	CD68+ population	0.20	0.042
	Histological inflammation severity	0.37	0.000
	Carcinogenesis progression	–0.27	0.009
CD86 protein expression	IFN-γ	0.23	0.022

3.3. *Lamina propria mononuclear cells populations in the UC and in the non-inflammatory carcinogenesis pathways*

LPMCs were similarly distributed along the UC and the non-inflammatory carcinogenesis pathways. T and B lymphocytes infiltrated significantly higher levels of lamina propria in the UC patients compared to those in the healthy subjects ($p = 0.006$ and $p = 0.033$, respectively). Patients with UC and dysplasia had significantly higher levels of monocyte CD68+ve infiltrating the lamina propria than did patients with



		Kendall's t	p-level
T cells (CD3+)	CEA serum levels	0,37	0,036
	NI carcinogenesis's progression	0,40	0,002
	IL2	0,40	0,000
	CD20	0,57	0,000
	CD68	0,59	0,000
	histological inflammation severity	0,59	0,000
B cells (CD20+)	NI carcinogenesis's progression	0,41	0,002
	IL1b	0,33	0,003
	CD68	0,57	0,000
	CD3	0,57	0,000
	histological inflammation severity	0,52	0,000
monocytes (CD68+)	CEA serum levels	0,63	0,000
	NI ccrcinogenesis's progression	0,39	0,003
	IL2	0,44	0,000
	CD20	0,57	0,000
	CD3	0,59	0,000
	histological inflammation severity	0,43	0,000

Fig. 2 – Lamina propria T cells, B cells and monocytes population in the colonic mucosa at the different steps of carcinogenesis and their significant correlations.

adenomas ($p = 0.039$). The significant correlations of lamina propria mononuclear cell populations in the UC and in the non-inflammatory carcinogenesis pathways are shown in Fig. 2.

3.4. *The cytokine network in UC and in the non-inflammatory carcinogenesis pathways*

IL-1β, IL-2 and IFN-γ mucosal expressions in the different step of UC and non-inflammatory carcinogenesis pathways and their significant correlation with carcinogenesis progression step and immune environment are illustrated in Fig. 3. IL-1β, IL-2 and IFN-γ mucosal expressions were directly correlated, in fact, with non-inflammatory carcinogenesis progression. IL-1β was, moreover, directly correlated with p53 expression in UC and non-inflammatory carcinogenesis. It was also correlated with IL-2 and IFN-γ mucosal expressions and clinical and histological inflammatory severity. IL-2 mucosal levels were not only correlated with histological disease severity and IL-1β mucosal expression but also with the CD80 protein expression.

4. Discussion

Infiltrating T cells, the main promoters of immunosurveillance, are found in many malignancies, but they do not appear to attack the tumour and seem to be mostly anergic, presumably because of the absence of activating and/or costimulatory signals.[29] In fact, antigen presenting cells infiltrating colorectal carcinomas, which express class II MHC, do not express costimulatory CD80 and CD86 molecules.[19] CD80 costimulatory molecules engineered in vaccines, which also encode genes for the carcinoembryonic antigen, have, nevertheless, been demonstrated to effectively enhance the immune response against colorectal cancer *in vivo*.[29,30] Why then does this mechanism seem to fail in colon carcinoma? On the basis of our data, the immunosurveillance mechanism elicited by CD80 expression on professional (LPMC) and non-professional antigen presenting cells (epithelial cells) is active in UC patients at the dysplastic stage. In fact, CD80 protein expression was significantly higher in our patients with UC and dysplasia compared to those with UC alone, while it was not significantly higher in the subjects with UC and cancer. Mucosal CD80 mRNA levels showed a similar, even if not statistically significant, tendency. Some other indications, such as CD80 up-regulation by human carcinoma cell lines induced by an oncogenic insult[22] and observed in the early stages of tumourigenesis[21] as well as in patients with colonic dysplasia[23], already seem to be pointing in that direction. Our finding that CD80 expression returns to baseline levels in patients with UC and colon cancer confirms the hypothesis, expressed in our previous study[23] that CD80 down-regulation can be associated to the progression from dysplasia to invasive cancer.[20] Moreover, we observed that CD80 protein, but not CD80 mRNA, expression was directly correlated with mucosal IL-2 expression suggesting that CD80 expression stimulates the production of this cytokine which is the main activating signal for T cell populations. According to these

		Kendall's τ	p-level
IL1β	p53 expression	0,22	0,033
	non inflammatory carcinogenesis' progression	0,49	0,001
	UC and non inflammatory carcinogenesis' progression	0,25	0,007
	IL2	0,25	0,006
	IFN γ	0,29	0,001
	histological inflammation severity	0,32	0,003
IL2	CD80 protein expression	0,21	0,034
	non inflammatory carcinogenesis' progression	0,38	0,008
	IL1β	0,25	0,006
	histological inflammation severity	0,34	0,002
IFN γ	CD86 protein expression	0,23	0,022
	non inflammatory carcinogenesis' progression	0,56	0,000
	IL1β	0,29	0,001

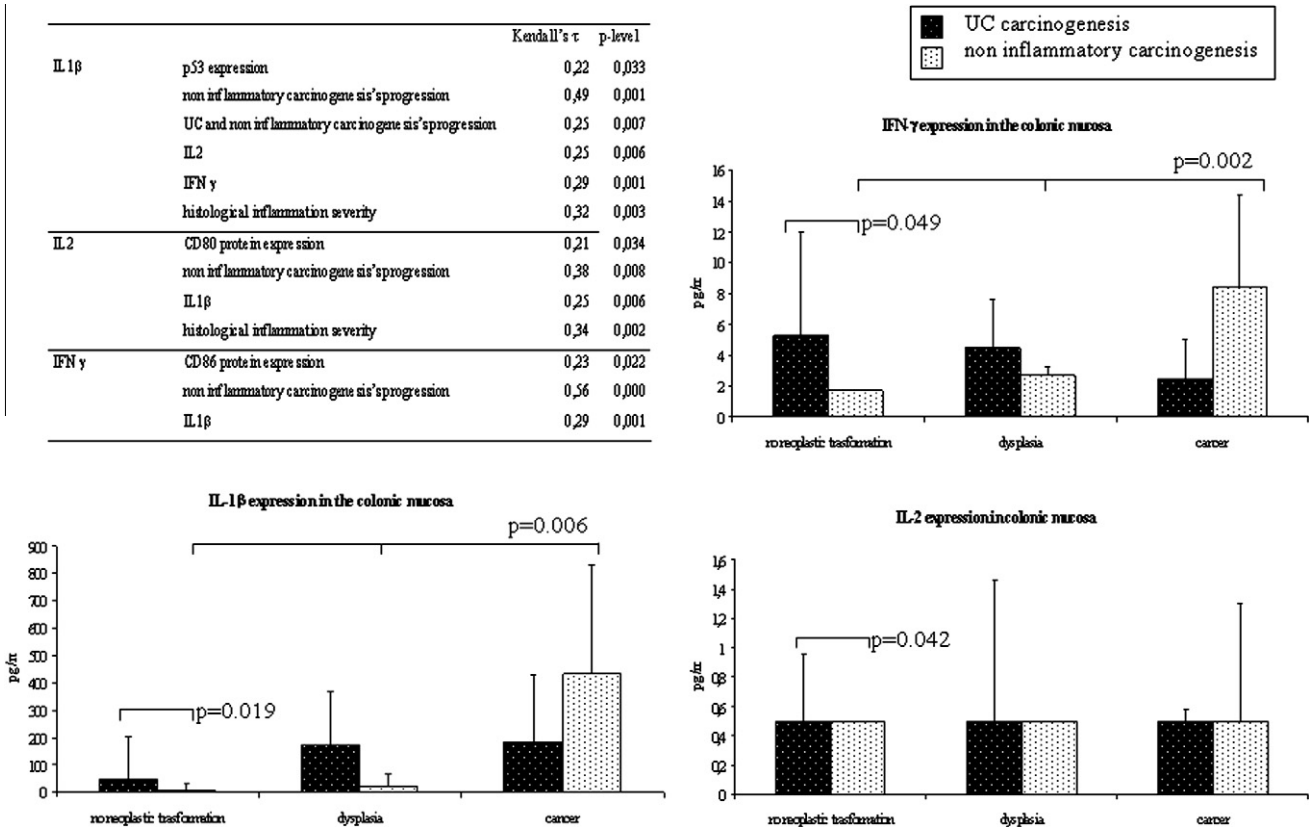


Fig. 3 – IL-1β, IL-2 and IFN-γ expressions in the colonic mucosa at the different steps of carcinogenesis and their significant correlations.

indications and our results, it would seem that there is a significantly higher CD80 expression in the dysplastic colonic tissue in UC while it is down-regulated in cancer and that that CD80 expression is directly correlated to mucosal T cell activation. This mechanism, however, does not seem to work when non-inflammatory colorectal carcinogenesis is at play. In fact, while CD80 does not seem to be constitutively expressed at all in the colonic mucosa of healthy subjects, its over-expression has never been reported in patients with non-inflammatory dysplasia.

The expression of CD80 and mRNA in non-inflammatory colonic carcinogenesis appeared to be lower to that in the corresponding steps in the UC pathway. CD80 protein expression in patients with UC and dysplasia was significantly higher than that in patients with colonic dysplastic adenoma. Although epidemiological data seem to suggest that adenoma prevalence results from a dynamic process including both adenoma formation and regression[31], some prospective studies with adequate follow-up times and adenoma site definition have failed to show complete regression or spontaneous reduction of polyps.[32,33] The lack of CD80 expression, probably due to the different carcinogenetic pathway involved, may explain why dysplastic adenomas persist once they have developed. An alternative or complementary hypothesis is that the up-regulation of CD80 and CD86 costimulatory molecules in the UC inflammatory activation described in several studies,[34–36] and also confirmed by the data presented herein, can be secondarily involved in the activation of this peculiar immunosurveillance mechanism.

The role of CD80 and CD86, however, seems to be different. In fact, CD86 protein or mRNA expression was significantly higher in UC patients than in the corresponding steps in patients with non-inflammatory carcinogenesis. There was a marked correlation between CD86 mRNA and histological and clinical inflammatory severity. Finally, CD86 expression appeared to be progressively down-regulated along the carcinogenesis process, while it seemed to be enhanced in UC colonic mucosa where it plays a role in the pathogenesis of inflammatory bowel disease.[34–36]

In conclusion, our study demonstrated that there is a significantly higher expression of CD80 in dysplastic colonic tissue in UC, while it seems to be down-regulated in cancer. CD80 mucosal levels correlate with the T cell population in the colonic mucosa and with the IL-2 expression suggesting that it has an active role in the immunosurveillance mechanism that prevents the progression of dysplastic foci in UC. But while CD80 does not seem to be constitutively expressed in the colonic mucosa of healthy subjects, CD80 over-expression has never been observed in patients with non-inflammatory dysplasia. The lack of CD80 expression may, then, explain the persistence of all dysplastic adenomas once they have developed.

Conflict of interest statement

None declared.

Disclosures

Preliminary data from this study were presented as a poster at the Digestive Disease Week which was held between 1 and 5 May 2010 in New Orleans, LN, USA and as posters at the 5th Annual Congress of the European Crohn's and Colitis Organization, which was held between 5 and 7 February 2010 in Prague, Czech Republic.

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