Inhibition of NF-κB activation and chemokine expression by the leukocyte glycoprotein, CD43, in colon cancer cells

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Abstract. CD43 is a heavily *O*-glycosylated type I transmembrane protein, expressed at high levels on the surface of leukocytes. It is frequently overexpressed in early colon adenomas, but not in normal colon epithelial cells. To identify CD43 target genes, gene array analysis was performed using a tetracycline-inducible CD43 expression system in human colon adenocarcinoma SW480 cells. CD43 was demonstrated to down-regulate a variety of chemokine genes. Overexpression of CD43 suppressed constitutive as well as PMA-induced NF-κB activation and reduced the DNA binding of transcription factor p65 but not p50. Furthermore, a reduced NF-κB responsive promoter activity was observed and a decreased expression of proinflammatory chemokines MCP-1, IL-8 and GRO-α. These results suggest that overexpression of CD43 suppresses a subset of NF-κB target genes, partly via the inhibition of p65 transcriptional activity.

Introduction

Cancer cells are known to express aberrant cell surface glycoproteins, such as MUC1 and CD44, suggested to have a role during cancer development by altering tumor cell adhesion, signaling and affecting immune surveillance. Another such molecule is CD43 (leukosialin, sialophorin), which under normal conditions is expressed on the surface of almost all hematopoietic cells (1). First, high CD43 expression was found in the colon cancer cell line, COLO205, and later at various levels in other intestinal cancer cell lines, such as Caco-2, HT-29, DLD-1 and SW480 (2). Moreover, CD43 expression was observed in a range of human tumors of epithelial origin (3). In particular, aberrant CD43 expression was found in early stages of human colorectal tumors but not in the normal colon epithelium (4,5), suggesting a role in colon tumor development.

CD43 is a type I transmembrane molecule. It has a mucin-like highly *O*-glycosylated extracellular domain with an extended structure (6). The intracellular part of CD43 is evolutionarily conserved and has no intrinsic catalytic activity but contains a number of potential protein kinase C (PKC) phosphorylation sites and an SH3-binding consensus sequence (6,7), suggesting that the intracellular domain of CD43 is involved in signal transduction. In hematopoietic cells, CD43 is ubiquitously expressed, but its multiple and complex functions remain controversial. However, CD43 has been demonstrated to be involved in regulation of cell adhesion (both pro- and anti-adhesion), immune responses, proliferation, survival and apoptosis.

The CD43 glycoprotein plays an important role in immune regulation. Differences in *O*-glycosylation give rise to different CD43 isoforms affecting the development, survival and reactivity of blood cells. For example, resting T lymphocytes express predominantly a 115-kDa isoform of CD43; however, upon activation more complex carbohydrate structures appear on CD43 resulting in a 130-kDa form (8). In T cell activation, both positive and negative regulatory roles have been ascribed to CD43. Several reports suggest that CD43 serves as a co-stimulatory receptor for T cell activation and show that the intracellular part of CD43 is involved in the formation of signal transduction complexes (9,10). On the contrary, T cells from CD43-deficient mice showed enhanced cytotoxic T cell responses and a delay in the down-modulation of immune responses, suggesting a negative regulatory role for CD43 (11,12). Besides, CD43 is actively excluded from immunological synapse by the ezrin-radixin-moesin (ERM) family of proteins allowing T cell activation (13). CD43 has been implicated to play a positive role in T cell homing to secondary lymphoid organs and peripheral tissue (14). Alterations in CD43 glycosylation in T cells have been linked to two unrelated human immunodeficiency diseases, Wiskott-Aldrich syndrome (WAS) (15) and acquired immune deficiency syndrome (AIDS) (16).

In tumor cells, CD43 has been speculated to interfere with immune cell interactions by making them more inefficient (17) and protect cells from Fas-induced cell death (18). However, CD43 probably has an even more diverse function and it can directly affect tumor cell signaling as indicated by its interaction with the oncogene ß-catenin (19). Moreover, CD43 overexpression, as a potential oncogenic stimulus, caused activation of the tumor suppressor proteins, p53 and ARF (20).

As CD43 expression has been found in colon adenomas, we have now asked how CD43 overexpression affects colon cancer cells to reveal how CD43 contributes to adenoma and maybe tumor development. We provide evidence that CD43

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overexpression inhibits NF-κB transcriptional activity and reduces DNA binding of the transcription factor, p65. Thereby, it may suppress the expression of genes involved in the regulation of immune responses.

Materials and methods

Cell culture. The human colon adenocarcinoma cell line, SW480, and the mammary gland adenocarcinoma cell line, MCF7, were cultivated in Iscove's medium containing 10% fetal bovine serum (Gibco-BRL) or 10% Tetracycline System Approved FBS (BD), and 1% penicillin-streptomycin (Invitrogen). To generate SW480/TR and MCF7/TR cell lines the pcDNA6/ TR regulatory plasmid of the tetracycline-inducible (tet-on) expression system, T-REx (Invitrogen), was transfected and selected by 10 μg/ml Blasticidin. Human full-length CD43 cDNA was cloned into the pcDNA4/TO expression plasmid, transfected into cells with the TR regulatory plasmid and stable clones selected by 50 μg/ml Zeocin. Protein overexpression was induced by 0.5-0.75 μg/ml tetracycline.

Gene chip microarray. Total RNA was extracted from 38 h tet-on and non-induced SW480/CD43 cells using an RNeasy mini kit (Qiagen), converted to cDNA, and hybridized to human U133 A/B chips (Affymetrix) by SweGene Microarray Centre (Lund, Sweden).

RT-PCR. For RT-PCR experiments, the cells were induced for indicated times and the total RNA extracted using the RNeasy mini kit (Qiagen). For RT-PCR the ProSTAR® HF Single-Tube RT-PCR system (Stratagene) was used with a final volume of 50 μl, containing 200 ng RNA and 10 pmol each primer. The primers used were: GRO-α (forward 5'-CA CCCCAAGAACATCCAAAG-3'; reverse 5'-TGCTCAAAC ACATTAGGCACA-3'), IL-8 (forward 5'-TCTCAGCCCTC TTCAAAAACTTCT-3'; reverse 5'-ATGAACTTCCAAGCT GGCCGTGCT-3'), and MCP-1 (forward 5'-CACTCACTCC ACAACCCAAG-3'; reverse 5'-GCAAAGACCCTCAAAAC ATC-3'). The primers for ß-actin were obtained from Applied Biosystems. For the PCR, 2.5 U Taq DNA polymerase (Invitrogen) was used and the reaction was conducted using GenAmpPCR system 9600 (Perkin-Elmer) at 42˚C for 30 min, followed by 95˚C for 1 min. The RT product was amplified by 20, 25 and 30 cycles of 95˚C (30 sec), 60˚C (30 sec) and 68˚C (2 min). The amplified fragments were resolved by 2% agarose gel electrophoresis and visualized with ethidium bromide.

Antibodies, Western blot analysis and immunocytochemistry. Two mAbs against human CD43 were used; αCD43-L10 reacting with the extracellular domain (21) and αCD43-4D2 with the intracellular domain of CD43 (22). The monoclonal antibodies for p65 and IκBα were from BD, and PPAR-γ and rabbit polyclonal anti-ß-catenin were from Santa Cruz Biotechnology. The polyclonal p50 antibody was from Upstate Biotechnology and ß-actin AC-15 antibody was from Sigma.

Cytoplasmic and nuclear extracts were prepared using NE-PER™ nuclear and cytoplasmic extraction kit (Pierce) according to the manufacturer's protocol, except for 3 additional washing steps with 0.1% NP-40 in PBS before nuclear lysis.

Complete EDTA-free protease inhibitor mixture (Roche) was added to the lysis buffers. Equal amounts of extracts, as determined by the BCA protein assay (Pierce), were analyzed by 10% homogenous SDS-polyacrylamide gel, transferred electrophoretically onto Immobilon P (Millipore), blocked with PBS-0.1% Tween-20 containing 5% milk powder, and probed with primary antibody diluted in block solution. Secondary antibodies (alkaline phosphatase or horseradish peroxidase conjugated, Dako) were diluted 1:2000 and developed with BCIP/NBT (Sigma) or SuperSignal West Pico (Pierce).

For p65 shuttling experiments, the cells were treated for 24 h with 10 ng/ml leptomycin B (Sigma). Alexa Fluor 488 anti-mouse IgG (Molecular Probes) was used as a secondary antibody for light microscopy.

Flow cytometry. Approximately 10^6 cells were washed with PBS and resuspended in FACS solution (1% BSA in PBS). For intracellular staining, the cells were kept on ice for 30 min with 0.05% saponin containing FACS solution. Primary antibody (2 μg/ml) was added to the cells and incubated on ice for 1-2 h. The cells were washed twice with FACS solution followed by centrifugation at 200 x g for 5 min. The cells were incubated with 1:100 diluted fluorescein-conjugated anti-mouse antibody (Dako) in FACS solution on ice for 45 min and washed as above. The samples were analyzed using a FACScalibur™ cell sorter (BD).

Chemokine ELISA. Spent cell culture media were collected and the level of IL-8, MCP-1 and GRO-α measured using colorimetric ELISA kits (R&D). The concentrations are given in pg/ml of the collected cell culture media.

Transfections and luciferase reporter assays. The cells were transiently transfected with DMRIE-C (Invitrogen) or Fugene6 (Roche) according to the manufacturer's protocol. The NF-κB-luc reporter plasmid was a kind gift from Dr Ian Whitehead. The pAP-1-luc plasmid was obtained from PathDetect AP-1 *cis*-Reporting System (Stratagene). The ß-catenin reporter system, pTOPFash, was used as described by (19). For the transfections, 1.5x10^5 cells were seeded in triplicates to 12-well plates 24 h before transfection. For transfection, 1 μg of AP-1-luc and NF-κB-luc reporter plasmids were used. The cells were treated for 24 h with 100 ng/ml PMA (Sigma). The cells were harvested 24 h after transfection with Passive Lysis buffer, and the luciferase activity determined (Promega Luciferase Assay). Relative light units (RLU) were measured using a Lumat 9501 luminometer (Berthold). All transfections were normalized to the level of total cellular protein by the BCA protein assay (Pierce). The results were analyzed by two-tailed unpaired t-test using the SPSS statistical package, and the level of significance was taken as $p < 0.05$.

NF-κB DNA binding activity. Nuclear extracts were obtained using a BD™ TransFactor extraction kit. The NF-κB DNA binding activities in nuclear extracts (35 μg) were quantified employing a Mercury™ TransFactor NF-κB family and p65 kit (BD).

RNAi. For p65 RNAi, 7x10^5 cells were transfected with 0.5 μg siRNA (SMARTpool NF-κB p65/RelA, Upstate Biotech-

nology) using the RNAi human/mouse control kit reagents (Qiagen). As a control, Alexa Fluor 488-labeled non-silencing siRNA was used. At 70 h after transfection, cells and medium were harvested and processed for Western blotting and MCP-1 ELISA.

Results

Overexpression of CD43. As a model to study the effect of observed CD43 expression in colon adenomas (4), a CD43 or lacZ tetracycline-inducible system was established in the colorectal adenocarcinoma cell line, SW480. Since CD43 expression was also found in breast carcinomas (3), the same system was also set up in the mammary gland adenocarcinoma cell line, MCF7. The different cells are referred to as SW480/CD43, MCF7/CD43, SW480/lacZ and MCF7/lacZ, whereas MCF7/TR cells only express the tetracycline repressor protein. Both studied cell lines, however, express low levels of endogenous CD43 (2). To verify CD43 overexpression after 48 h of tetracycline induction (48-h tet-on), the cells were studied by FACS using mAb αCD43-4D2 against the cytoplasmic tail of human CD43 (Fig. 1A). An approximate 4-fold increase in the expression of CD43 was observed in SW480/CD43 cells, whereas tetracycline induction of MCF7/CD43 cells caused higher expression levels of CD43 compared to SW480 cells. The level of CD43 expression achieved after induction in SW480/CD43 cells is comparable with the endogenous CD43 expression levels in the human leukemic cell line, K562, and the colorectal adenocarcinoma cell line, COLO205 (2). SW480/CD43 cells were further analyzed by Western blotting using mAb αCD43-4D2 (Fig. 1B). The observed masses of CD43 are similar to those described previously (23). After 24-h tet-on, the precursor band of CD43 was found at approximately 54 kD, and the glycosylated mature forms as a smear between 110 and 150 kD, which further increased in amounts after 48-h tet-on. To verify the cell membrane localization of over-expressed CD43, non-permeablised cells were stained with mAb αCD43-L10, reacting with an extracellular epitope on CD43. An approximate 3.5-fold increase in CD43 surface expression was observed in the SW480/CD43 cells by FACS (Fig. 1C). The localization of CD43 to the cell membrane was almost exclusive as shown by immunohistochemistry using mAb αCD43-4D2 in the permeablilized clone of SW480/CD43 (Fig. 1E). This localization was previously also shown by confocal microscopy (2). It should be emphasized that the expression level of CD43 in the non-induced SW480/CD43 cells was higher than in the SW480/lacZ cells, due to the general problem of this system with incomplete repression of this inducible promoter (Fig. 1D).

CD43 alters the gene expression in cancer cells. To identify genes whose mRNA expression level had changed upon CD43 overexpression, the cDNA from non-induced and 38 h tet-on SW480/CD43 cells were analyzed on the human Affymetrix gene array. Using a >3-fold difference as criterion of differential expression, 18 genes were identified (Table I). Only four genes were up-regulated upon CD43 overexpression including an apoptosis-related serine/threonine kinase 17a (DRAK1). The identities of the others are unknown and have not been studied further. Instead, most of the affected genes
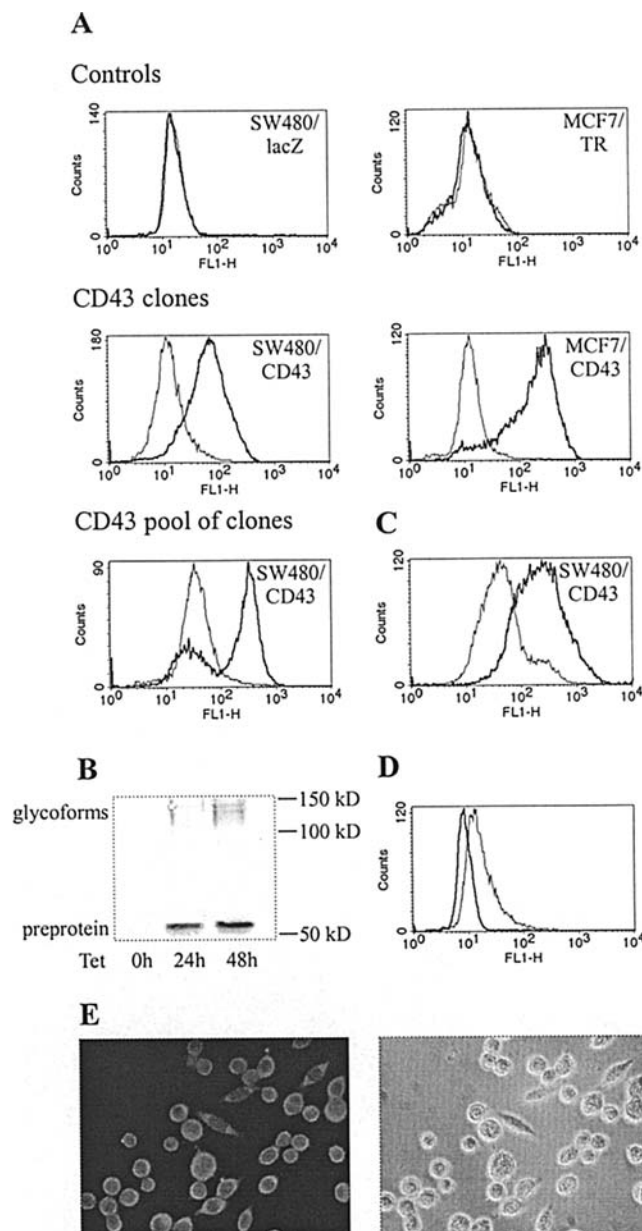

Figure 1. Tet-on overexpression of CD43 and lacZ in the colon adeno-carcinoma cell line, SW480, and the mammary gland adenocarcinoma cell line, MCF7. (A) FACS analysis of intracellular CD43. Permeabilized cells were stained with mAb αCD43-4D2 against the cytoplasmic domain of CD43, followed by FITC-conjugated secondary anti-mouse antibody. Thin line, non-induced cells; bold line, 48-h tet-on cells. (B) Western analysis of 24-, 48-h tet-on SW480/CD43 cells using mAb αCD43-4D2. The preprotein of CD43 migrated as a 54-kD band, and glycosylated mature forms as a smear between 110 and 150 kD. (C) FACS analysis of the cell surface expression of CD43 in non-permeablised 48-h tet-on SW480/CD43 cells, using mAb αCD43-L10 reacting with the extracellular domain of CD43, followed by FITC-conjugated anti-mouse antibody. (D) Comparison of CD43 expression between SW480/lacZ (bold line) and non-induced SW480/CD43 (thin line) cells. The cells were cultivated using 10% Tetracycline System Approved FBS and stained with mAb αCD43-4D2 followed by FITC-conjugated anti-mouse antibody. (E) Localization of CD43 in 48-h tet-on SW480/CD43 cells as studied by light fluorescence microscopy. The cells were stained with mAb αCD43-4D2, followed by Alexa488-conjugated secondary anti-mouse antibody.

showed decreased expression, including several chemokines belonging to the CXC and CC families. The MCP-1 (CCL2) expression decreased 4-fold and the other chemokines, such

Table I. Gene expression alterations upon overexpression of CD43 according to Affymetrix gene chip U133

Affymetrix ID	GenBank ID	Description	Fold change
Down-regulated			
215069_at	AK025065	N-myristoyltransferase 2 (NMT2)	6.4↓
242518_at	AA748423	Hypothetical protein LOC90408	5.8↓
209328_x_a	BC000587	Hypothetical protein HSPC111	4.4↓
233703_x_at	AK024236	cDNA FLJ14174 fis, clone NT2RP2002843	4.4↓
216598_s_at	S69738	Chemokine (C-C motif) ligand 2 (CCL2, MCP-1)	4.0↓
222180_at	AU147889	cDNA clone FLJ14122 fis, clone MAMMA1002033	3.8↓
227509_x_at	W18193	cDNA FLJ40982 fis, clone UTERU2014601	3.8↓
210748_at	AF116696	DnaJ (Hsp40) homolog, subfamily C, member 8	3.8↓
207798_s_at	NM_017492	Ataxin 2 related protein (A2LP)	3.4↓
231819_at	BG505096	cDNA clone IMAGE:5261836, partial cds	3.4↓
202859_x_at	NM_000584	Interleukin 8 (IL-8)	3.0↓
204470_at	NM_001511	Chemokine (C-X-C motif) ligand 1 (CXCL1, GRO-α)	3.0↓
210941_at	AB006756	Protocadherin 7 (PCDH7. BH-Pcdh)	3.0↓
242260_at	BG283790	Matrin 3 (MATR3)	3.0↓
207850_at	NM_002090	Chemokine (C-X-C motif) ligand 3 (CXCL3, GRO-γ)	2.4↓
207339_s_at	NM_002341	Lymphotoxin ß (LTß)	2.4↓
210317_s_at	U28936	Epsilon 14-3-3	2.4↓
209774_x_at	NM_002089	Chemokine (C-X-C motif) ligand 2 (CXCL2, GRO-ß)	2.2↓
208075_s_at	NM_006273	Chemokine (C-C motif) ligand 7 (CCL7, MCP-3)	2.2↓
Up-regulated			
243378_at	BE673747	cDNA clone IMAGE: 3278205	5.2↑
242255_at	R49102	KIAA0982 protein	4.0↑
242161_at	AI458049	cDNA clone IMAGE:2146521 (STK17A)	4.0↑
236705_at	AA017245	Hypothetical protein MGC42090	3.4↑

as GRO-α (CXCL1/MGSA), and IL-8 (CXCL8) decreased 3-fold. Moreover, three chemokines, MCP-3 (CCL7), GRO-ß (CXCL2) and GRO-γ (CXCL3), showed a more than 2-fold down-regulation upon CD43 overexpression (Table I). Elevated CD43 levels reduced the expression of N-myristoyltransferase 2 (NMT2), ataxin 2-related protein (A2LP), protocadherin 7 (PCDH7), nuclear matrix protein matrin 3 (MATR3), DNAJC8 (DnaJ, a Hsp40 homolog, subfamily C, member 8) and 14-3-3 epsilon. The latter has been previously shown to be repressed by CD43 (24).

To confirm some of the gene array results, the mRNA expression levels of chemokines GRO-α, IL-8, and MCP-1 were further studied by semi-quantitative RT-PCR. The results confirmed decreased expression of GRO-α, MCP-1 and IL-8 upon CD43 overexpression for 44-h tet-on (Fig. 2A). To exclude clonal effects, GRO-α, MCP-1 and IL-8 mRNA levels were also studied in the SW480/CD43 pool of transfectants.

CD43 suppresses the production of proinflammatory chemokines. To further investigate if CD43 inhibits the production of chemokines, the levels of GRO-α, MCP-1 and IL-8 were analyzed in the cell culture medium of SW480/CD43 and SW480/lacZ cells by ELISA. MCP-1 secretion was reduced

3.6-fold whereas GRO-α and IL-8 secretion were reduced 3-fold after 48-h tet-on in the SW480/CD43 cells, showing a good correlation with the cDNA array results. In the control cells, no major changes in the levels of secreted chemokines were observed (Fig. 2B). Overexpression of CD43 reduced the transcription of IL-8 in SW480/CD43 cells (Fig. 2A), but both the mRNA and protein levels of IL-8, in contrast to GRO-α and MCP-1, were higher in non-induced SW480/CD43 cells as compared to SW480/lacZ cells. This suggests that the elevated CD43 expression in the non-induced SW480/CD43 cells (Fig. 1D) may cause increased IL-8 levels. In T cells, CD43 has been shown to enhance IL-8 production (25). The inhibition of chemokine production correlated with the reduced mRNA levels indicating that CD43 can regulate the transcription of proinflammatory molecules.

CD43 interferes with the transcriptional activity of NF-κB. One of the main modulators of genes involved in the control of immune and inflammatory responses, including chemokines and cytokines, is the nuclear factor κB (NF-κB) family of transcription factors. Due to the fact that elevation of CD43 levels decreased the expression of several NF-κB target genes, such as GRO-α, MCP-1 and protocadherin 7, we investigated
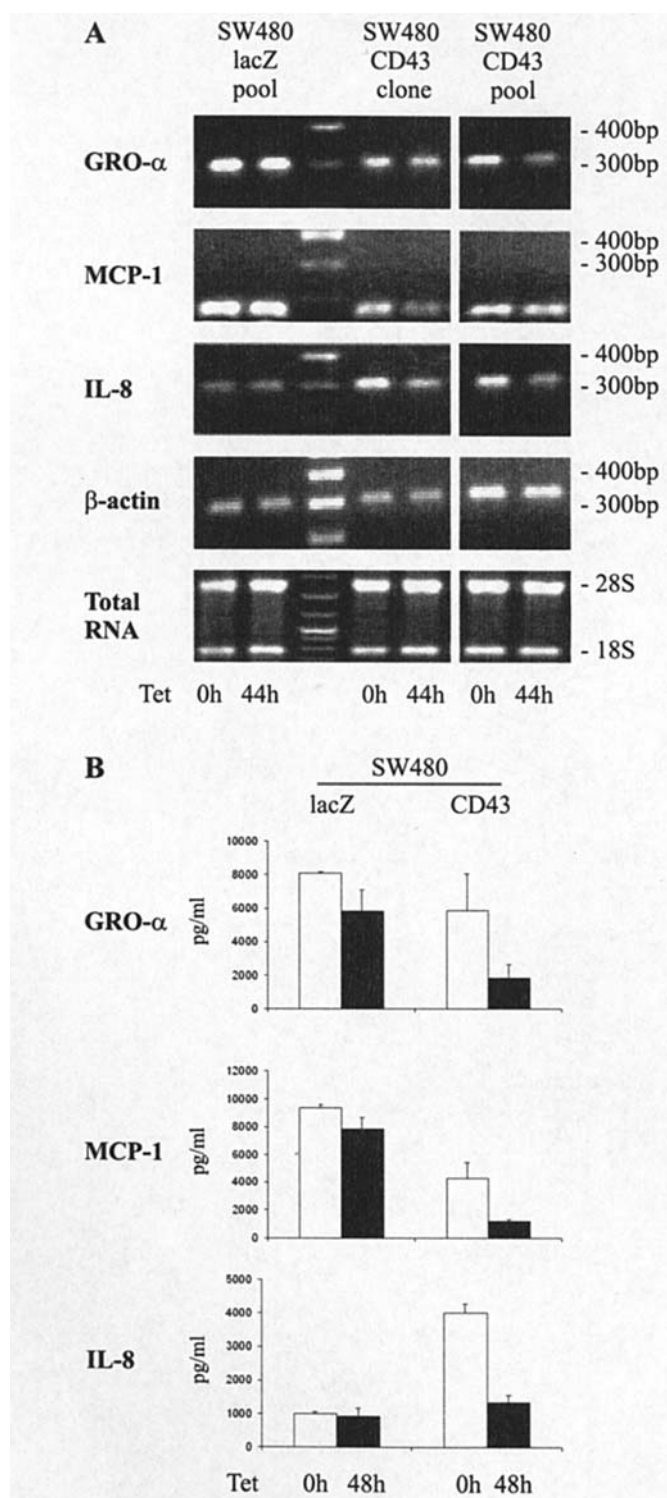
Figure 2. Overexpression of CD43 lowers mRNA expression and protein secretion of the chemokines, GRO-α, MCP-1 and IL-8. (A) Total RNA was extracted from the 44-h tet-on SW480/lacZ and SW480/CD43 pool of cells and from the SW480/CD43 clone, reverse transcribed and subjected to RT-PCR by using primers specific for ß-actin, GRO-α, MCP-1 and IL-8. Sub-saturating levels of RT-PCR products (cycle 25) were compared using 2% agarose gel electrophoresis. Total RNA (1 μg) was also analyzed revealing equal amounts. (B) The levels of chemokines GRO-α, MCP-1 and IL-8 were determined after 48-h tet-on in the SW480/CD43 and SW480/lacZ cell culture medium by ELISA (pg/ml). The results represent the mean ± SD of two independent experiments performed in duplicate.

whether the transcription factor, NF-κB, could be one of the regulators of their expression in the studied cells. NF-κB target promoter activation upon CD43 overexpression was tested using a luciferase reporter. The SW480 cells were transiently transfected with NF-κB luciferase reporter plasmid and the relative luciferase activity of non-induced and 24-h tet-on cells was compared. The promoter activity was significantly reduced in the CD43 overexpressing cells compared to control cells (Fig. 3A). This suggests that the observed inhibition of chemokine transcription can be mediated by reduced transcriptional activity of NF-κB. However, in some experiments, it was noticed that the basal level of NF-κB luciferase activity was higher in SW480/CD43 cells than in SW480/lacZ cells (data not shown), indicating that low levels of CD43, probably due to promoter leakage, may increase NF-κB activity. This could explain the observed higher IL-8 secretion in the non-induced SW480/CD43 cells. Next, we tested the CD43-dependent inhibition of NF-κB in phorbol ester (PMA)-activated cells. PMA stimulation for 24 h increased the expression of the NF-κB promoter dependent reporter by approximately 2-fold. In SW480/CD43 cells, CD43 overexpression significantly reduced NF-κB promoter activity whereas control cells did not show any alterations (Fig. 3A). As NF-κB is known to cooperate with other transcription factors, such as activating protein 1 (AP-1) and ß-catenin, luciferase reporter assays were used to study the target promoter activity. Transfected and PMA-treated 24-h tet-on SW480/lacZ and SW480/CD43 cells did not show any significant changes of AP-1 (Fig. 3B) or ß-catenin (data not shown) responsive transcriptional activity upon CD43 induction.

CD43 suppresses the nuclear localization and DNA binding activity of NF-κB p65. There are five known mammalian NF-κB family members: p65 (RelA), RelB, c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100), which can form homo- or heterodimers. In most cell types, the inactive NF-κB complex, commonly composed of p65 and p50 subunits, is located in the cytoplasm bound to their inhibitors, IκBs. Upon activation by different stimuli, IκB kinase complex (IKK) is activated, resulting in the phosphorylation of IκB, which targets IκB to proteolytic degradation. Removal of this inhibitory complex allows active NF-κB dimers to translocate to the nucleus, bind specific DNA motifs called κB sites, and activate target gene transcription (26). The NF-κB pathway is regulated by many processes, including IKK activity, post-translational modifications as well as nucleocytoplasmic shuttling of various components of the pathway.

At first we asked whether CD43 could interfere with p65 and p50 localization to the nucleus by subjecting separated nuclear and cytoplasmic fractions to Western blot analysis. In unstimulated SW480 cells, the majority of p65 and p50 were located in the cytoplasm, but with detectable amounts also in the nucleus. When the nuclear fractions were analyzed, 15-, 27- and 51-h tet-on SW480/CD43 cells showed reduced amounts of p65, but SW480/lacZ cells did not (Fig. 3C). With longer CD43 induction times, a time-dependent gradual decrease of p65 in the nucleus was revealed, whereas the amount of nuclear p50 was not affected. As the total amount of p65, measured by FACS, remained the same at 48-h tet-on CD43 (Fig. 3D), it probably did not affect the turnover of p65.

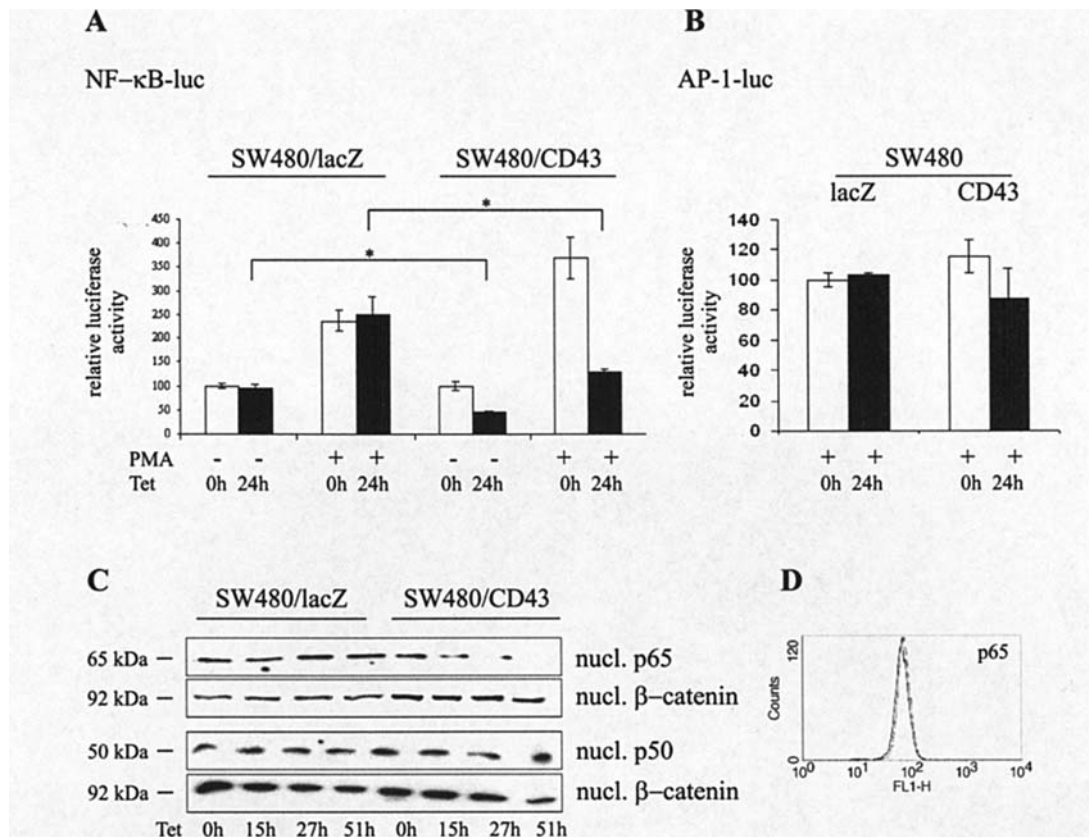To determine the DNA binding activity of the NF-κB family, an ELISA-like assay was used. It employs immobilized

Figure 3. CD43 inhibits NF-κB transcriptional activity in SW480 cells. (A) A plasmid encoding an NF-κB responsive luciferase reporter (1 μg of NF-κB-Luc) was transiently transfected into SW480/lacZ and SW480/CD43 cells, and the relative luciferase activities were measured 24h after transfection. Simultaneously, the cells were 24-h tet-on, and stimulated with 100 ng/ml PMA. Relative luciferase activity is expressed as the percentage relative to tetracycline non-induced and non-PMA treated cells. All results were normalized to total protein determined with the BCA protein assay and presented as mean and standard error of the mean of two independent experiments performed in triplicate ($^{*}p<0.05$, t-test). (B) AP-1-responsive promoter activity was determined by luciferase reporter assay in 100 ng/ml PMA-stimulated SW480/lacZ and SW480/CD43 cells. The cells were 24-h tet-on induced, transfected with 1 μg AP-1-luc reporter plasmid and stimulated with 100 ng/ml PMA and 24 h later followed by the analysis of relative luciferase activity. (C) CD43 suppresses nuclear translocation of NF-κB p65. The cytoplasmic and nuclear extracts were separated, and the nuclear (nucl.) fraction analyzed by Western blotting after normalization of the total protein with the BCA protein assay. Western blot analysis of p65, p50 and ß-catenin in the nuclear fraction of 15-, 27- and 51-h tet-on SW480/lacZ and SW480/CD43 cells showed reduced amounts of p65 in the nucleus upon CD43 overexpression, whereas nuclear p50 and ß-catenin levels were unaffected. (D) FACS analysis of NF-κB p65 in permeabilised 48-h tet-on SW480/CD43 cells.

dsDNA corresponding to κB sites to capture NF-κB complexes and specific antibodies to detect bound transcription factors. The nuclear extracts of 24-h tet-on SW480/CD43 cells showed reduced p65 DNA binding compared to control cells (Fig. 4A), correlating with reduced amounts of p65 in the nucleus. The DNA binding of the other NF-κB family members (RelB, c-Rel) were not affected or showed no DNA binding (p50, p52), but the p65 binding activity was reduced already at 16-h tet-on (Fig. 4B).

To confirm that transcription factor p65 is involved in the regulation of chemokine expression in our system, SW480/lacZ cells were transfected with p65 siRNA and, as a control, non-silencing siRNA. The cells were lysed 70 h after transfection and the cell culture medium was analyzed by MCP-1 ELISA. The p65 silenced cells showed decreased secretion of MCP-1 compared to the control cells, suggesting that MCP-1 expression is dependent on p65 in SW480 cells (Fig. 4C).

In order to confirm the inhibition of NF-κB by CD43 in different cell lines, NF-κB target promoter activation was also studied in MCF7 cells. Similarly to SW480/CD43 cells, 24-h tet-on MCF7/CD43 cells showed a significant reduction in basal NF-κB promoter activity as well as after 24-h PMA

stimulation (Fig. 5A). Moreover, CD43 overexpression also decreased the nuclear p65 in MCF7 cells (Fig. 5B), showing that the effect was not restricted to colon cancer cells.

To study whether CD43 could interfere with constitutive nucleocytoplasmic shuttling of p65, we treated 24-h tet-on SW480/CD43 and MCF7/CD43 cells with leptomycin B (LMB), a selective inhibitor of chromosomal region maintenance-1 (CRM-1)-dependent nuclear export. Immuno-fluorescence studies of p65 in the presence of LMB for 24 h showed, as expected, that p65 localized almost exclusively in the nucleus. However, overexpression of CD43 in SW480 and MCF7 did not interrupt the nuclear accumulation of p65 after inhibition of nuclear export by LMB (data not shown). Recently, PPAR-γ was shown to enhance the nuclear export of p65 (27). To address whether CD43 overexpression could enhance the nuclear export of p65 via PPAR-γ, the localization of PPAR-γ was studied. However, CD43 overexpression did not cause any cytoplasmic relocalization of PPAR-γ (Fig. 6A).

Finally, we examined whether CD43 interferes with IκBα degradation after stimulation with TNFα (15, 30 or 60 min), which is a known potent proinflammatory cytokine and activator of NF-κB. TNFα-induced NF-κB activation is
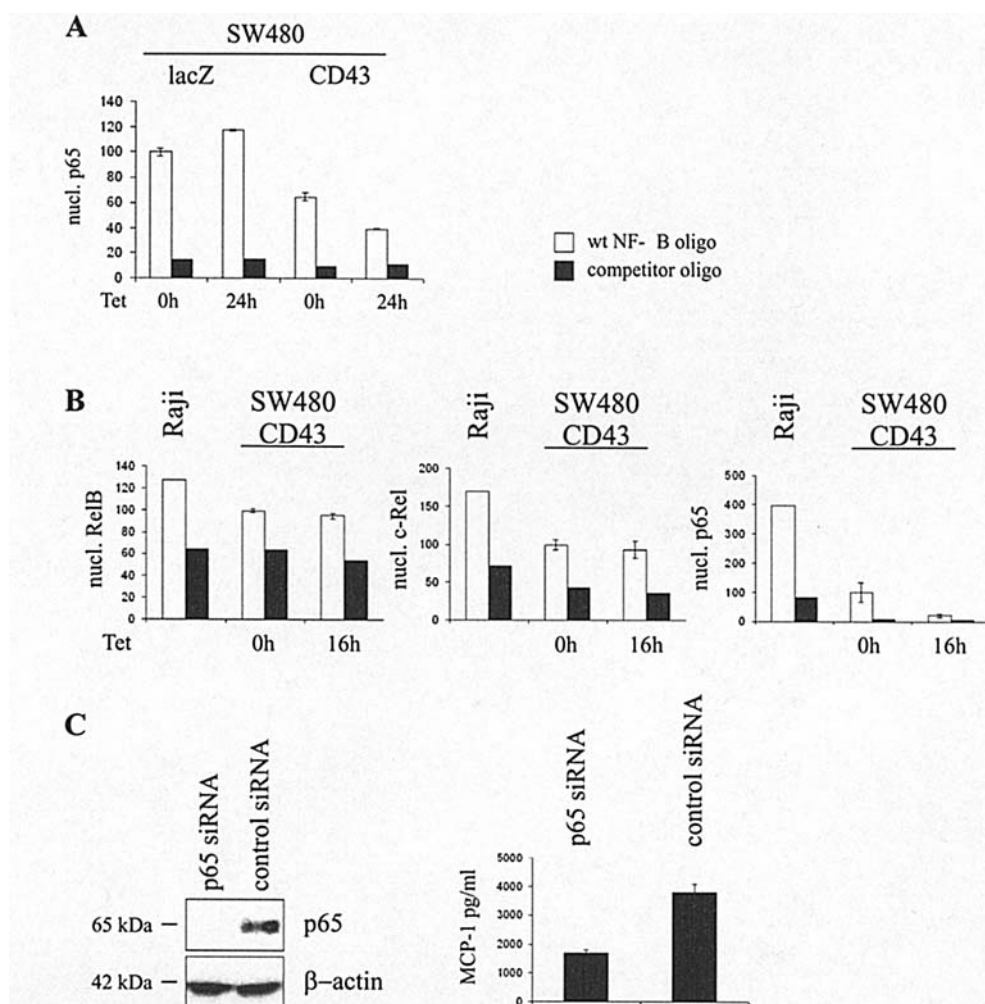
Figure 4. CD43 inhibits DNA binding of NF-κB p65. Nuclear lysates of SW480/lacZ and SW480/CD43 cells were prepared and NF-κB DNA binding activities determined by an ELISA-based assay. Wells with immobilized wild-type (wt) κB element-containing DNA oligonucleotides and with 250 ng of wt κB element-containing the appropriate oligonucleotide in the solution (competitor) are shown. (A) p65 DNA binding activity of 24-h tet-on SW480/lacZ and SW480/CD43 cells. Results are presented as percentage of the means of absorbance (OD 650 nm ± SD of duplicate wells in one out of three representative experiments) relative to non-induced SW480/lacZ cells. (B) RelB, c-Rel and p65 DNA binding activities in nuclear lysates of 16-h tet-on SW480/CD43 cells. Cellular extract from Raji cells was used as a positive control. (C) p65 RNAi reduces MCP-1 production in the SW480 cells. SW480/lacZ cells were analyzed by Western blotting 70 h after transient transfection with p65 and control siRNA. The media were analyzed for MCP-1 by ELISA (pg/ml).

characterized by IKK-mediated IκBα phosphorylation and degradation (26). In 30-min TNFα-treated cells, increased nuclear translocation of p65 compared to non-stimulated cells was observed (data not shown). Upon CD43 expression 24-h tet-on, SW480/CD43 cells again showed less p65 in the nucleus (Fig. 6B). TNFα treatment for 30 min caused complete IκBα degradation in SW480/lacZ cells whereas SW480/CD43 cells showed slower degradation of IκBα (Fig. 6C).

Discussion

A high expression of the leukocyte-associated antigen, CD43, has been found in human colorectal adenomas (4). The prevalence of CD43 expression in early stages of colon adenomas, not in normal colon, and variable expression in advanced colon carcinoma suggests a role during early colon tumor development. To study the potential functions of CD43 in cancer cells, a tetracycline inducible overexpression of CD43 in adenocarcinoma cell lines with low endogenous expression was established. In the studied colon adenocarcinoma cell line, SW480, overexpression of CD43 suppressed the transcription factor p65 activity and down-regulated the expression of pro-inflammatory genes, indicating that CD43 may play a negative role in the regulation of immune responses.

CD43 is an immunoregulatory molecule involved in seemingly contradictory functions as it can regulate T cell adhesion, homing, and activation both positively and negatively. The extracellular domain of CD43 can be differently glycosylated, directly affecting the immune function (28). However, the intracellular domain of CD43 is also important for immune regulation as it is involved in signal transduction. Ligation of CD43 with mAb causes PKC activation and triggers the MAP kinase pathway recruiting AP-1, NF-κB and NF-AT transcription factors (29). In contrast, ligation of CD43 with a mAb against another glycoform repressed the nuclear localization of NF-κB (30). It is thus likely that different CD43 glycoforms have different effects on signal transduction.

In the present study, we show that CD43 overexpression in cancer cells inhibits NF-κB activation, probably by reducing the DNA binding activity of transcription factor p65 and not

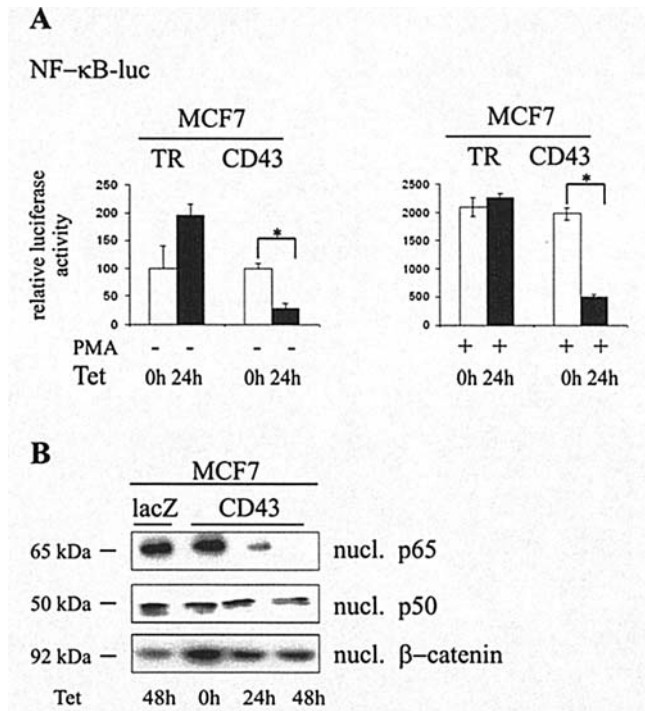Figure 5. CD43 inhibits NF-κB in MCF7 cells. (A) NF-κB responsive luciferase reporter plasmid was transfected into MCF7/lacZ and MCF7/CD43 cells and the relative luciferase activities were measured 24 h after trans-fection. The cells were induced as indicated with tetracycline and stimulated with 100 ng/ml PMA for 24 h. The results are normalized to total protein determined with the BCA protein assay and presented as mean and standard error of the mean of two independent experiments performed in duplicate (*p<0.05, t-test). (B) Western blot analysis of nuclear p65, p50 and ß-catenin in 24- and 48-h tet-on MCF7/CD43 cells showing decreased amount of nuclear p65.

Figure 6. (A) Western blot analysis of PPAR-γ in the nuclear and cyto-plasmic fraction of 48-h tet-on SW480 cells. CD43 did not induce trans-location of PPAR-γ from the nucleus to the cytoplasm. (B) SW480/lacZ and SW480/CD43 cells were treated with 10 ng/ml TNFα for 30 min, and the nuclear fractions were analyzed by Western blotting using p65, p50 and ß-catenin antibodies. CD43 overexpressing cells showed smaller amounts of nuclear p65 whereas nuclear p50 and ß-catenin levels were unaffected upon TNFα treatment. (C) Kinetics of TNFα induced IκBα degradation in SW480/lacZ and SW480/CD43 cells. The 0-, 6- and 24-h tet-on SW480/lacZ and SW480/CD43 cells were treated with TNFα for 15, 30 and 60 min, and Western blot analysis was performed with IκBα, and ß-catenin antibodies.

p50, which may be dependent on slower IκBα degradation. The inhibition of p65 activity was linked to the lower secretion of the proinflammatory chemokine, MCP-1, in SW480 cells. The promoters of MCP-1, and IL-8 genes are known to be dependent on transcription factor p65/p65 and p65/p50 dimer complexes for activation (31). This suggests that CD43 may regulate chemokine expression via suppression of p65 DNA binding activity. It has also been reported that expression of IL-8 and MCP-1 is dependent on ß-catenin (32,33). As we have previously shown that the cytoplasmic tail of CD43 can localize to the nucleus and interact with ß-catenin when expressed as a stabilized protein by fusion with GFP, the present results might depend on ß-catenin as well as NF-κB (19). However, overexpression of full-length CD43 in the present cell lines did not show any nuclear localization suggesting that the observed effects of CD43 overexpression are more likely due to the cytoplasmic and membrane localized interference of NF-κB signaling. We did not observe any transactivation effect of CD43 on a ß-catenin responsive promotor reporter (data not shown), but it cannot be excluded that the observed effects might be linked to ß-catenin. Importantly, ß-catenin was shown to form a complex with NF-κB, reduce its DNA binding, and repress target gene expression in human colon and breast cancer cells (34).

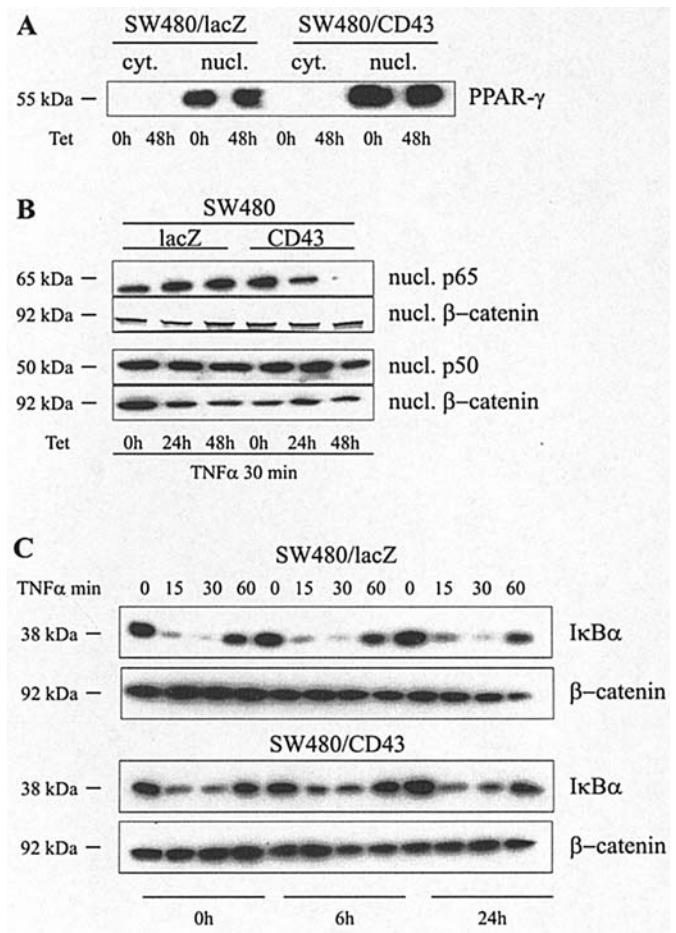As CD43 overexpression caused inhibition of NF-κB, we speculate that tumor cells expressing CD43 may suppress immune responses via inhibition of chemokine expression. The biological significance of chemokines in the tumor micro-environment is complex. Chemokines can influence the extent of infiltration and phenotype of the immune cells entering the tumors, affect angiogenesis and tumor cell growth, survival and migration. Some of the chemokines have a dual and biphasic effect on tumor growth. One such example is MCP-1, an important determinant of macrophage infiltration into tumors. Low levels of MCP-1 secreted by melanoma cells gave modest monocyte infiltration, whereas high levels invoked massive macrophage infiltration and tumor destruction (35). Thus, high expression levels of CD43 that can suppress chemokine secretion may represent a mechanism to lower the infiltration of immune cells into a developing tumor. In later stages of colon carcinogenesis, chemokines might positively contribute to tumor growth. In fact, CD43 expression is significantly reduced in later stages of colon carcinoma development (4).

It is commonly believed that NF-κB activation contributes to tumor progression. Increased activity of NF-κB is detected in many cancer cell lines derived either from hematopoetic or solid tumors, as well as in tumor tissue of different origins.

However, the role of the NF-κB pathway in oncogenesis tends to be cellular context dependent as several recent observations also suggest NF-κB contribution to pathways inhibiting cancer development. Inhibition of NF-κB in skin cells was linked to the development of squamous cell carcinoma (36,37). Furthermore, depletion of NF-κB p65 resulted in a malignant phenotype of embryonic fibroblasts (38) and overexpression of NF-κB p65 in MCF7 cells reduced tumorigenicity in nude mice (39). In colon cancer cell lines, expression of the oncogenes, ß-catenin and Ras, was shown to decrease the NF-κB p65 activity (34,40). Thus, in certain situations, CD43 may contribute to oncogenesis by inhibiting NF-κB p65. Moreover, NF-κB activity is modulated by the tumor suppressors, p53 and ARF, and it is proposed that NF-κB might be tumor suppressing rather than tumor promoting in early stages of cancer (41). Importantly, CD43 causes activation of p53 via the tumor suppressor protein, ARF (20). When these tumor suppressor proteins were active, NF-κB p65 was demonstrated to repress rather than activate anti-apoptotic gene expression (42). Thus, CD43 expression in tumor cells may have a complex contribution to cell fate during tumor development. The phenotypic effects and biological significance of CD43 expression in early colon tumor development might be dependent on CD43 dosage as well as the status of tumor suppressor proteins.

In brief, overexpressed CD43 suppressed NF-κB p65 in the studied cancer cell lines. However, further experiments are necessary to explain more precisely how CD43 mediates this effect on NF-κB signaling, information that should help us to understand how CD43 could be involved in early colon tumor development.

Acknowledgements

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