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CD40 Ligand-CD40 Interaction Induces Chemokines in Cervical Carcinoma Cells in Synergism with IFN-γ[1]

Andreas Altenburg,* Stephan E. Baldus,† Hans Smola,‡ Herbert Pfister,* and Sigrun Hess[2]*

Cellular immunity plays a major role in controlling human papilloma virus infection and development of cervical carcinoma. Mononuclear cell infiltration possibly due to the action of chemokines becomes prominent in the tumor tissue. In fact, the macrophage chemoattractant protein-1, MCP-1, was detected in cervical squamous cell carcinoma in situ, whereas absent in cultured cells. From this, unknown environmental factors were postulated regulating chemokine expression in vivo. In this study, we show high CD40 expression on cervical carcinoma cells and CD40 ligand (CD40L) staining on attracted T cells in tumor tissue, suggesting a paracrine stimulation mechanism via CD40L-CD40 interactions. We therefore investigated chemokine synthesis in nonmalignant and malignant human papilloma virus-positive cell lines after CD40L exposure. Constitutive expression of MCP-1, MCP-3, RANTES, and IFN-γ-inducible protein-10 was almost undetectable in all cell lines tested. CD40L was able to induce MCP-1 production; however, despite much higher CD40 expression in malignant cells, MCP-1 induction was significantly lower compared with nontumorigenic cells. After sensitization with IFN-γ, another T cell-derived cytokine showing minimal effects on CD40 expression levels, CD40 ligation led to a more than 20-fold MCP-1 induction in carcinoma cell lines. An even stronger effect was observed for IFN-γ-inducible protein-10. Our study highlights the synergism of T cell-derived mediators such as CD40L and IFN-γ for chemokine responses in cervical carcinoma cells, helping to understand the chemokine expression patterns observed in vivo. ***The Journal of Immunology,*** **1999, 162: 4140–4147.**

Mononuclear cell infiltration is observed frequently in cervical carcinoma and has been suggested to play an important role in host defense (1). In neoplastic lesions, immune cells are recruited by soluble chemotactic factors originating from carcinoma cells, and the fate of the lesions is eventually determined by a complex interaction between the attracted immune cells and the carcinoma cells.

Chemotactic cytokines are classified as CXC chemokines (α-chemokines) or CC chemokines (β-chemokines), depending on whether or not the first two cysteines are separated by one amino acid. CXC chemokine family members, i.e., the prototype IL-8 (2, 3) or growth-stimulatory activity-α, -β, and -γ, are primarily responsible for the recruitment of neutrophils. Exceptions are CXC chemokines such as IP-10[3] (IFN-γ-inducible protein) and MIG (monokine induced by IFN-γ), which may act on T lymphocytes. CC chemokines, i.e., the prototype MCP-1 (4, 5), MCP-2 to -4, RANTES, and MIP-1α and -β, preferentially attract monocytes, T lymphocytes, eosinophils, and NK cells (reviewed in Ref. 6). Synthesis of chemokines can occur in many cell types after stimulation with proinflammatory cytokines, particularly IFN-γ, IL-1, and members of the TNF family, including CD40L (7). However, which of these proinflammatory mediators are primarily responsible for the induction of chemokines in cervical carcinomas is as yet unresolved.

CD40, the receptor for CD40L, is a member of the TNFR family. It is expressed on B cells, monocytes (8, 9), dendritic cells (10), and a variety of nonhemopoietic cells, including normal keratinocytes (11), tumor cells, and many in vitro transformed and carcinoma-derived cell lines (12–15). CD40L (gp39), a type II transmembrane protein with homology to TNF, has been shown to be transiently induced on $CD4^+$ T cells upon activation (16). The interaction between CD40L and CD40 is not only crucial in B cell differentiation and activation (reviewed in Ref. 17), but is also important for different inflammatory responses of nonhemopoietic cell types (18). Examples are the induction of chemokines such as IL-8 in skin keratinocytes and MCP-1 in renal cells (7, 19) and the production of IL-6 (20), the latter being a growth factor for a variety of cell types including cervical carcinomas (21).

As a central signaling mechanism, CD40 induces the translocation of NF-κB (22), a transcription factor regulating many genes such as IL-6, TNF, and MHC class I and MHC class II controlling inflammatory responses (23). Three intracellular proteins, TNFR-associated factor (TRAF) 2, TRAF5, and TRAF6, that associate with the cytoplasmic domain of CD40, were shown to mediate NF-κB activation (24–26). At least TRAF2 and TRAF6 involve the mitogen-activated protein 3 kinase-related kinase NIK (27), a pathway that is shared with the TNF receptor. In fact, NF-κB is also involved in the regulation of both prototypes of chemokine families, namely IL-8 (28) and MCP-1 (29, 30).

In vivo, MCP-1 is markedly expressed in cervical squamous cell carcinomas (SCCs) with an inflammatory reaction. In situ hybridization and immunohistochemical studies revealed that MCP-1 transcripts and protein predominated in neoplastic epithelial cells (31). This contrasts with the in vitro situation, in which cervical carcinoma cell lines, e.g., HeLa, were reported to express only

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[3] Abbreviations used in this paper: IP-10, IFN-γ-inducible protein; BHK, baby hamster kidney; CD40L, CD40 ligand; CIN, cervical intraepithelial neoplasia; EMSA, electrophoretic mobility shift assay; HPV, human papilloma virus; MCP-1, monocyte-chemoattractant protein-1; NF-κB, nuclear factor-κB; pAb, polyclonal Ab; SCC, squamous cell carcinoma; SN, supernatant; TRAF, TNFR -associated factor wt, wild type.

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extremely low MCP-1 mRNA levels (32, 33). It therefore was anticipated that additional environmental factors might exist that up-regulate chemokines in cervical carcinoma cells in vivo (31).

Our initial studies have shown high expression of CD40 in cervical carcinoma and of CD40L in adjacent mononuclear cells in vivo. Hence, we asked the question as to whether CD40 activation might be involved in the regulation of chemokine production in HPV-positive epithelial cells. We provide evidence that two synergistic stimuli, CD40L- and IFN-γ-dependent signals, are sufficient for efficient chemokine induction in cervical carcinoma cells in vitro. These results might explain the expression pattern of chemokines in cervical SCC observed in vivo.

Materials and Methods

Cells

The HPV16- or HPV18-positive cervical carcinoma cell lines SiHa (ATCC HTB-35), CaSki (ATCC CRL-1550), and SW756 (kindly provided by Dr. M. von Knebel-Doeberitz, Heidelberg, Germany), and the HPV16-transformed foreskin keratinocyte cell line HPK IA (34) and baby hamster kidney (BHK) cells were cultured in DMEM (Life Technologies, Eggenstein, Germany). The routine culture medium for BHK cells transfected with CD40L cDNA (BHK_{CD40L}) (35) contained 200 nM methotrexate (Sigma, Deisenhofen, Germany) and 100 μg/ml G418 (Life Technologies). All media were supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, and 2 mM L-alanyl-L-glutamine (all from Life Technologies). The in vitro HPV18 E6/7-transformed cell strains K51 and I56 (kindly provided by Dr. L. A. Laimins, Chicago, IL) were maintained in DMEM containing 25% Ham's F12 medium, 10% FCS, 50 μg/ml gentamicin, 0.4 μg/ml hydrocortisone, 10^{-10} M cholera toxin, 5 μg/ml transferrin, 2×10^{-11} M triiodthyronine, 1.8×10^{-4} M adenine, 5 μg/ml insulin (all from Sigma), and 10 ng/ml epidermal growth factor (Life Technologies).

Immunohistochemistry

Ten tissue specimens with cervical intraepithelial neoplasia (CIN) representing all grades of dysplasia (CIN I-III) and six specimens with SCC of the cervix derived from the files of the Institute of Pathology, University of Cologne (Cologne, Germany), routinely fixed in 5% Formalin and paraffin embedded, were investigated immunohistochemically. After deparaffinization, the slides were subjected to microwave treatment for 3×5 min at 750W in 0.1 M citrate buffer for enhanced Ag retrieval. Subsequently, the ImmunoMax technique (36), modifying the catalyzed reporter deposition technique (37), was applied. The primary mAbs anti-CD40 mAb G28-5 (38) and anti-CD40L mAb (PharMingen, Hamburg, Germany) were diluted in 10% normal mouse serum and incubated overnight at 4°C. Biotinylated rabbit anti-mouse Ab (E354; Dako, Carpinteria, CA) was added for 30 min at room temperature, followed by a peroxidase-coupled streptavidin-biotin complex (k355; Dako) for 30 min at room temperature. In the next step, biotinylated tyramine solution (20 mg of *N*-hydroxysuccinimido-sulfo-LC-biotin (Pierce, Seattle, WA) dissolved in 0.5 ml of DMSO allowed to react with 6.4 mg of tyramine (Sigma) in 10 ml of 0.1 M borate buffer, pH 8) was applied for 10 min at room temperature. This solution had been preincubated for 72 h at 4°C, readjusted to pH 8, and stored at −80°C. Before application, it was diluted 1/50 (v/v) in Tris-buffered saline (TBS)/0.05% H_2O_2. StreptAB-alkaline phosphatase complex (K391; Dako) was incubated for 30 min at room temperature. Between all steps, threefold washing with TBS was performed. Finally, the reaction products were visualized using naphthol-AS-biphosphate and new fuchsin as chromogens. Nuclei were counterstained with hematoxylin.

The subpopulations of inflammatory cells that could be recognized in all specimens, i.e., T lymphocytes as well as myelomonocytic cells, were characterized by routine immunohistochemistry (ABC alkaline phosphatase) using the mAbs directed against CD8 (DK25; Dako), CD15 (LeuM1; Becton Dickinson, Heidelberg, Germany), and CD68 (PG-M1; Dako). Only the mAb OPD4 (anti-CD45R0; Dako), detecting a helper/inducer phenotype of T cells, was applied using the above-described ImmunoMax technique.

FACS analysis

Cells either unstimulated or after stimulation with 1000 U/ml IFN-γ (Life Technologies) for 48 h were detached with PBS containing 5 mM EDTA (Sigma). Following blocking with 2% BSA (Sigma) in PBS, cells were incubated with 5 μg/ml anti-CD40 mAb (IgG1; PharMingen) or MOPC-21 (Sigma) as an isotype-matched control. Cells were then stained with FITC-conjugated goat anti-mouse $F(ab')_2$ (Dianova, Hamburg, Germany), and CD40 expression was determined by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA).

Induction of cytokine production

Cells were either used unstimulated or prestimulated with 1000 U/ml IFN-γ (Life Technologies) for 24 h. They were then seeded in 24-well plates at a density of 1.5×10^5 cells/well again in the absence or presence of 1000 U/ml IFN-γ, respectively. After 24 h, they were stimulated with BHK cells expressing CD40L, CD40L-negative BHK wild-type cells, or medium as a control in a 300-μl volume. BHK cells had been freshly detached with PBS containing 5 mM EDTA preserving CD40L expression on their surface. After 16 h, cellular supernatants (SN) were collected, centrifuged, and stored at −20°C.

Determination of cytokines by ELISA

Cytokine ELISAs were essentially done as described (20). Maxisorp plates (Nunc, Wiesbaden, Germany) were coated with 1 μg/ml anti-IL-6, anti-MCP-1, or anti-IL-8 mAb (PharMingen) or 2 μg/ml anti-RANTES mAb (Biosource, Fleurus, Belgium) overnight. After blocking of the plates for 1 h with PBS containing 0.5% BSA, 0.05% Tween 20 (Serva, Heidelberg, Germany), and 0.02% NaN_3, SN or serial dilutions of the respective recombinant human cytokines (Tebu, Frankfurt, Germany) as standards were added for 6 h. In the case of RANTES, 5% skim milk was used as blocking reagent. Plates were then incubated with anti-IL-6, anti-MCP-1 pAb, or anti-IL-8 pAb at 0.5 μg/ml or anti-RANTES pAb at 1 μg/ml overnight (all pAbs were purchased from Tebu). For the detection of MCP-3 and IP-10, concentrations of the capture Abs were 5 and 2 μg/ml, and for the biotinylated detection Abs 3 μg/ml and 500 ng/ml, respectively (PharMingen and R&D, Wiesbaden, Germany). After 2-h incubation with peroxidase-labeled goat anti-rabbit $F(ab')_2$, or streptavidin-peroxidase (Dianova) in the case of MCP-3 and IP-10, the substrate was applied and the extinction was measured with a SLT ELISA reader at 405 nm.

Electrophoretic mobility shift assay (EMSA)

Cervical carcinoma cell lines were grown in 10-cm culture plates overnight to subconfluency. For CD40 stimulation, paraformaldehyde-fixed BHK_{CD40L} or BHK_{wt} cells were used. These cells were prepared as described (39). Briefly, BHK cells were detached with 5 mM EDTA in PBS, fixed with 3% paraformaldehyde, and washed six times with PBS. The cervical cell lines were then stimulated either with medium or 2.4×10^7 BHK cells. After 1.5-h incubation at 37°C, fixed BHK cells were removed, the carcinoma cells were harvested, and nuclear extracts were prepared according to Dignam et al. (40). After preincubation of the nuclear extracts containing 5 μg protein with poly(dI-dC), NF-κB-binding activity was examined. The extracts were incubated for 20 min at room temperature with a ^{32}P-labeled double-stranded 30-bp probe from the mouse κ light chain enhancer containing one NF-κB site (41, 42). In supershift experiments, the nuclear extracts were preincubated with the appropriate Abs (anti-p50 and anti-p65; Santa Cruz Biotechnology, Santa Cruz, CA) for 40 min before adding the probe.

Results

CD40 and CD40L are expressed in cervical carcinoma

In normal squamous epithelium of the cervix, CD40 was found only in cells of the basal layer (Fig. 1*a*). Specimens with different stages of dysplasia showed a similar pattern of reactivity regardless of the CIN grade (Fig. 1*b*). On the other hand, CD40 expression was strongly up-regulated in SCCs. An intense membrane-associated reactivity was observed, which was accompanied by a slighter, diffuse cytoplasmic Ag distribution in all parts of the tumors (Fig. 1*c*). However, neighboring intraepithelial neoplasia behaved as described for CIN above. Five of the six SCCs studied displayed a moderate to strong inflammatory reaction; the sixth tumor exhibited a strong one. Numerous macrophages were detected in the infiltrate, as revealed with mAb PG-M1, which binds a macrophage-restricted epitope of the CD68 Ag (Fig. 1*d*). Infiltrates of the SCCs also contained granulocytes and monocytes, as revealed by staining of the CD15 Ag. Moreover, both CD45R0- and CD8-expressing lymphocyte populations were present in the infiltrates surrounding tumor growth (not shown). CD40L was detected on a subpopulation of tumor-infiltrating lymphocytes (Fig.

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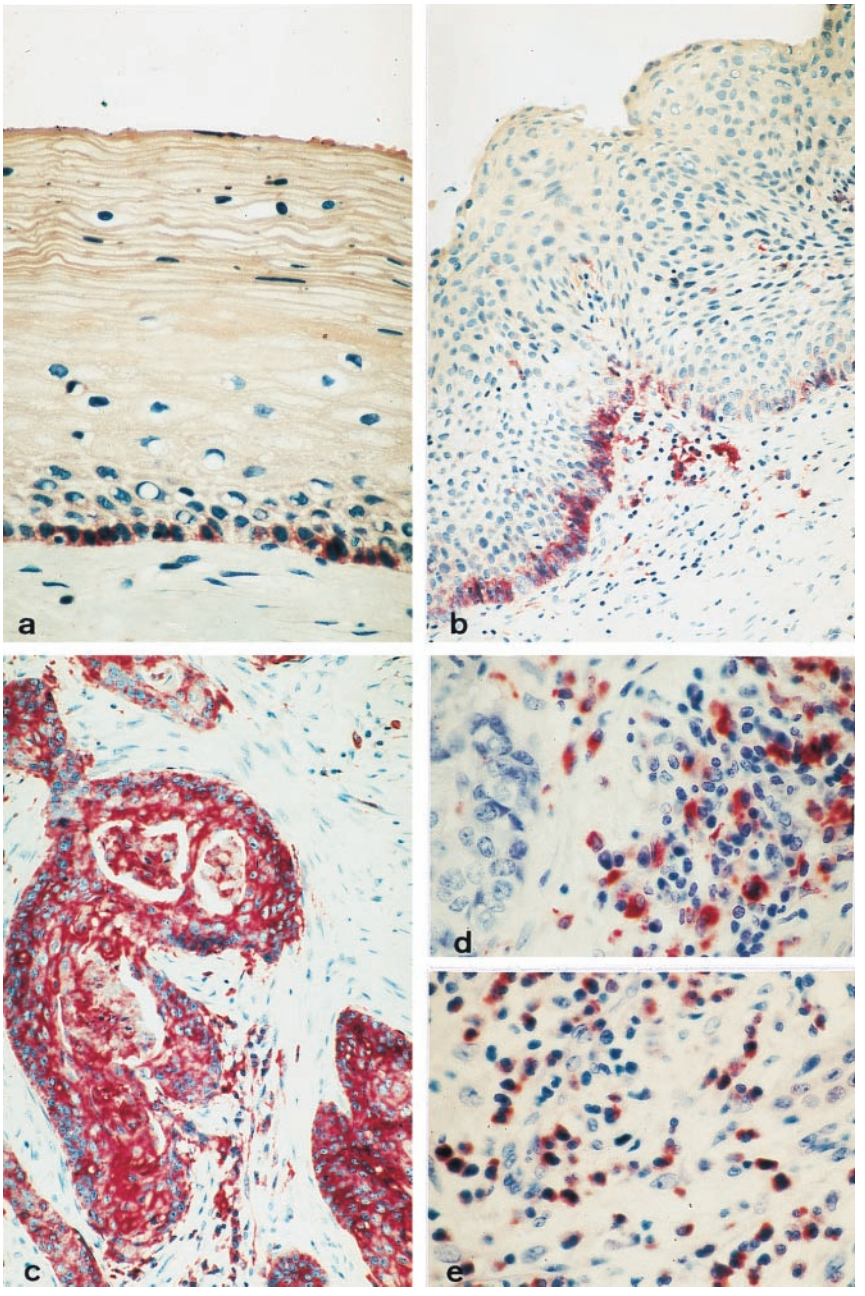

FIGURE 1. CD40 and CD40L expression in normal and neoplastic cervical epithelium in situ. CD40 was immunostained with anti-CD40 mAb G28-5 in normal cervical epithelium (*a*), CIN III lesion (*b*), and SCC (*c*) using the ImmunoMax technique, as described in *Materials and Methods*. Staining of macrophages (*d*) using the anti-CD68 mAb PG-M1, or CD40L (*e*) using an anti-CD40L mAb in the infiltrate of a SCC.

1*e*), providing the basis for a paracrine stimulation mechanism via CD40L and CD40 interactions in cervical SCC.

CD40 was also found on cervical cell lines cultured in vitro. In HPV-positive carcinoma cell lines (SiHa, SW756, CaSki), FACS analysis revealed a strong CD40 expression (Fig. 2, *lower panel*). Nontumorigenic keratinocytes that were transformed with HPV in vitro (I56, K51, HPK IA) showed only low basal CD40 expression (Fig. 2, *upper panel*) and, as with other cell types, CD40 expression was up-regulated by IFN-γ treatment (Fig. 2). Of these cells, HPK IA was the one that displayed the greatest up-regulation of CD40 expression in response to IFN-γ treatment.

Low constitutive MCP-1 production in HPV-positive cell lines

HPK IA cells representing nontumorigenic in vitro transformed keratinocytes were compared with the carcinoma cell lines SiHa and SW756 with respect to MCP-1 production. All three cell lines constitutively secreted only low levels of MCP-1 (less than 120 pg/ml) into the culture medium (Fig. 3, *upper panel*). The low MCP-1 production of cervical carcinoma cell lines in vitro contrasted to the up-regulation of MCP-1 in cervical SCC observed in vivo. This discrepancy might either be explained by suppressive factors present in culture, but not in vivo, by the lack of MCP-1 regulating intracellular signals in cultured cell lines or by additional stimulatory factors present in vivo, but not in vitro. Our analyses showed that TGF-β_1, which suppresses MCP-1 production at least in macrophages (43), was ruled out as a negative regulator of MCP-1 in our carcinoma cells, as the investigated cell lines did not produce any detectable amounts of TGF-β_1 (data not shown). Moreover, the transcriptional regulator SP-1, which is relevant for constitutive human MCP-1 gene expression (29), was prevalent in nuclear extracts of the cervical carcinoma cell lines according to gel-shift experiments with a SP-1 binding site-specific oligonucleotide (data not shown). We therefore speculated that in vivo

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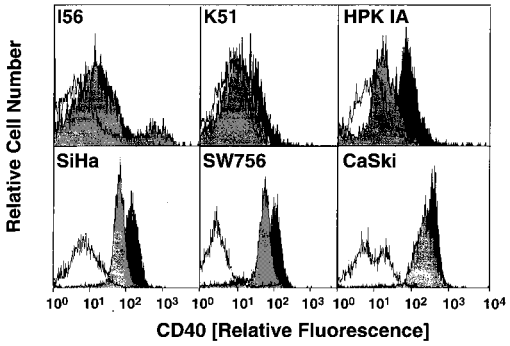

FIGURE 2. Papilloma virus-positive cervical carcinoma cell lines express CD40 at higher levels than in vitro transformed keratinocytes. For immunostaining with anti-CD40 mAb G28-5 (▒, ■) or the isotype-matched control mAb MOPC-21 (□), in vitro transformed cell lines (*top*) or cervical carcinoma cell lines (*bottom*) were either left untreated (▒) or pretreated with 1000 U/ml IFN-γ for 48 h (■). After incubation with FITC-conjugated goat anti-mouse Ab, CD40 expression was determined by flow cytometry.

environmental factors were present, leading to the up-regulation of MCP-1 in cervical carcinoma cells.

CD40 mediates MCP-1 production in in vitro transformed and carcinoma-derived cell lines

As CD40 was found to be highly expressed in cervical SCC, while CD40L was detected in the tumor infiltrate, functional experiments were performed to further analyze whether the CD40L-CD40 interaction could account for MCP-1 expression in cervical carcinoma. Previous work has shown that membrane-bound CD40L resulted in stronger stimulation of CD40 compared with soluble, proteolytically released CD40L (20). Therefore, CD40 was activated with CD40L-bearing BHK cells that did not interfere with any of the cytokine assays (data not shown). Stimulation of HPKIA cells with CD40L-bearing cells resulted in a more than ninefold induction of MCP-1 (up to 922 pg/ml) (Fig. 3*A*, *left panel*). BHK_{wt} cells did not alter MCP-1 production. Up-regulation of MCP-1 was also seen after stimulation with paraformaldehyde-fixed BHK cells, although their stimulatory capacity was weaker, as already described earlier (20). In contrast, SiHa and SW756 cells were significantly less efficient at producing MCP-1 after CD40 ligation. Indeed, SiHa and SW756 cell MCP-1 production reached maximums of 278 and 322 pg/ml, respectively (Fig. 3*A*, *middle* and *right panel*), this despite their higher CD40 expression levels.

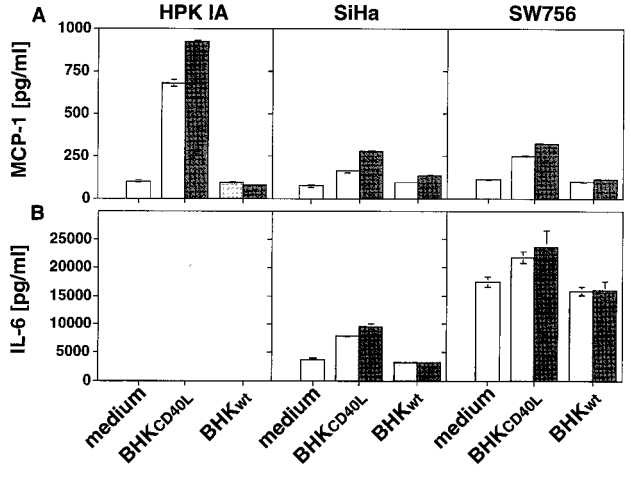

FIGURE 3. Comparison of MCP-1 (*A*) and IL-6 (*B*) induction by CD40 in HPK IA, SiHa, and SW756 cells. Cells seeded at equal densities (1.5×10^5 cells/well) were stimulated with medium (□), BHK_{CD40L}, or BHK_{wt} cells at 2×10^4 cells/well (▒), or 6×10^4 cells/well (■). Sixteen hours later, the SN were harvested and the MCP-1 or IL-6 levels were determined by ELISA. In all cases, the mean values of duplicate determinations of one representative experiment are shown. In cases in which SDs are not visible, these were below 1% of the mean values.

CD40 induces NF-κB-binding activity in cervical carcinoma cell lines

To obtain information on the mechanism of CD40-mediated MCP-1 induction, we investigated whether CD40 was able to activate the NF-κB transcription factor in cervical carcinoma cell lines. NF-κB had previously been shown to be crucially involved in MCP-1 regulation. Nuclear extracts prepared from SW756 showed weak constitutive NF-κB-like binding activity (Fig. 4*A*, *lane 4*). Membrane-bound CD40L strongly induced NF-κB-binding activity in both SW756 and SiHa (Fig. 4*A*, *lanes 2* and *5*), while BHK_{wt} cells did not (Fig. 4*A*, *lanes 3* and *6*). In both cell lines, two complexes (complex I and II) resulted in EMSA experiments. Supershift analysis using specific Abs recognizing the p50 or p65 subunits of NF-κB, respectively, revealed that complex I, which was strongly mobilized by CD40L, mainly contained the p65 and also p50 subunits of NF-κB. The much weaker complex II only contained p50/p50 homodimers (Fig. 4*B*). These results indicated that binding activity of NF-κB complexes is strongly induced by CD40L in cervical carcinoma cells, with a predominance of complex I probably representing functional p65/p65 homodimers and p50/p65 heterodimers.

Having considered the high surface CD40 expression on cervical carcinoma cells, the strong mobilization of NF-κB-binding activity, and the weak MCP-1 response after CD40 activation, one could speculate on a selectively reduced inducibility of MCP-1 in these cell lines. To put this hypothesis to the test, CD40-mediated induction of IL-6, a growth factor for cervical carcinoma, was investigated. This cytokine does not belong to the chemokine family, but the promoter of the IL-6 gene shares several regulatory elements, including binding sites for NF-κB responsible for transcriptional activation. We have reported recently that IL-6 may be induced by CD40 in non-B cells and that its induction correlates to the CD40 expression level within a cell line (20). When HPK IA cells were stimulated with CD40L, no IL-6 production was measurable (Fig. 3*B*, *left panel*), which could therefore not be compared with MCP-1 induction by CD40. SiHa cells produced up to 9.5 ng/ml of IL-6 after CD40 ligation, and IL-6 production after CD40 stimulation was even higher in SW756 cells reaching 23.6 ng/ml (Fig. 3*B*, *middle* and *right panels*). However, both of these cell lines also constitutively expressed high amounts of IL-6. Thus, the CD40-mediated inducibility of IL-6 in cervical carcinoma cells (1.3–2.4-fold) was of the same order as that of MCP-1 (2.8–3.5-fold), but took place on a much higher niveau. These experiments strongly argued against a selective unresponsiveness of the MCP-1 gene to inflammatory stimuli.

IFN-γ induces MCP-1 production in HPV-positive cervical keratinocytes

In vivo, CD40L may not represent the only stimulus responsible for MCP-1 induction in cervical carcinoma. Besides CD40L, activated $CD4^+$ tumor-infiltrating T cells may also produce high amounts of IFN-γ, a cytokine that is known to exert a regulatory action on some chemokines, including MCP-1 (44, 45). IFN-γ stimulation of SiHa cells raised the production of MCP-1 into the culture supernatants only to low levels (258 pg/ml). However, a

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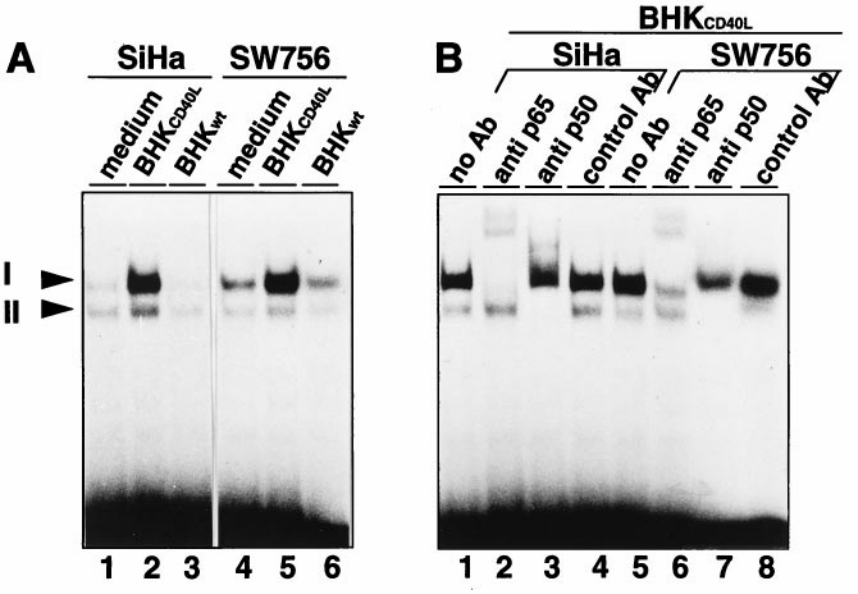

FIGURE 4. CD40 mobilizes NF-κB in the cervical carcinoma cell lines SiHa and SW756 (*A*). Cells were incubated for 1.5 h with medium (*lanes 1* and *4*), paraformaldehyde-fixed BHK_{CD40L} cells (*lanes 2* and *5*), or BHK_{wt} cells (*lanes 3* and *6*). Nuclear extracts were analyzed for NF-κB-binding activity by EMSA, as described in *Materials and Methods*. CD40-induced NF-κB complexes contain p65 and p50 NF-κB subunits (*B*). CD40-induced NF-κB binding complexes (*lanes 1* and *5*) were examined by supershift analysis using anti NF-κB p65 pAb (*lanes 2* and *6*), anti-NF-κB p50 pAb (*lanes 3* and *7*), or nonspecific rabbit Ig as a control (*lanes 4* and *8*). Arrows indicate the main complexes composed of p65-p65 homodimers or p65-p50 heterodimers (complex I) and p50 homodimers (complex II).

stronger response was seen with the tumorigenic cell line SW756. The latter showed an induction of MCP-1 to even higher levels than in HPK IA cells (1200 and 819 pg/ml, respectively) (Fig. 5).

IFN-γ sensitizes cervical carcinomas for CD40-mediated MCP-1 production

Stimulation of IFN-γ-preactivated cells with CD40L-bearing cells led to a strong increase in MCP-1 production not only in HPK IA cells, but also most notably in the tumorigenic cell lines SiHa and SW756. In the case of SiHa, up to 1600 pg/ml MCP-1, and in the case of SW756, up to 2800 pg/ml MCP-1 were produced (Fig. 5). Compared with stimulation with medium alone, the combined effect of IFN-γ preactivation and CD40L stimulation resulted in a more than 20-fold induction of MCP-1 production by SiHa cells, a 25-fold increase for SW756, and a 39-fold increase for HPK IA cells.

As shown in Fig. 2, IFN-γ stimulation also up-regulated CD40 expression in HPK IA cells and to a lesser extent in SiHa and SW756. To examine whether IFN-γ-preactivated carcinoma cells generally responded better to CD40 ligation, IL-6 was again taken as a "reference cytokine" and its production was measured. While in IFN-γ-preactivated HPK IA cells IL-6 could be induced at least to measurable amounts (72 pg/ml) after CD40 activation, IL-6 levels of CD40-stimulated SiHa and SW756 were not altered by IFN-γ preactivation (data not shown). These findings suggested that synergistic induction of MCP-1 after IFN-γ and CD40L stimulation is not simply due to the up-regulation of CD40, which should have influenced also the induction of other cytokines, i.e., IL-6, but takes place on the level of intracellular signaling.

IFN-γ sensitizes cervical carcinomas for CD40L-mediated CC-chemokine and IP-10, but not IL-8 production

To investigate whether the sensitization by IFN-γ was a specificity of MCP-1 regulation or a more general phenomenon, we measured the CD40L-mediated induction of other chemokines of the CC (RANTES and MCP-3) and CXC chemokine family (IL-8 and IP-10) combined with or without IFN-γ preactivation (Fig. 6).

RANTES and MCP-3 were produced only at very low levels in all three cell lines at the detection limit of the ELISA, which was approximately 40 pg/ml. CD40 stimulation alone did not significantly enhance MCP-3 production in SiHa and SW756 (Table I). However, IFN-γ stimulation clearly sensitized not only HPK IA, but also SW756 and to a lesser extent SiHa cells for the induction of both chemokines by CD40L (Fig. 6, *upper panel*).

A similar but much stronger effect was observed for IP-10, a CXC chemokine that attracts activated T lymphocytes (Fig. 6, *lower panel*). The basal quantities of IP-10 in the supernatants of all three cell lines were at the detection limit of the IP-10 ELISA (between 0 and 75 pg/ml), and CD40L stimulation could increase the IP-10 production only to low amounts (or not at all in the case of SW756 cells). However, high IP-10 levels were measured already after IFN-γ stimulation. Concentrations ranged from 5 ng/ml in SiHa cells, 30 ng/ml in HPK IA, to 38 ng/ml in SW756 cells. When these IFN-γ-sensitized cells were stimulated with CD40L, a strong synergism was measured for IP-10 production, reaching up to 106 ng/ml in HPK IA, 66 ng/ml in SiHa, and 65 ng/ml in SW756 cells.

A regulation pattern different from that seen for the above-mentioned chemokines was observed for IL-8, a chemokine that predominantly attracts neutrophils to sites of inflammation (Fig. 6, *lower panel*). IL-8 was induced more than 4.5-fold in HPK IA as well as in SiHa cells after CD40 ligation and to a lower degree in SW756 cells. IFN-γ, rather, had a negative than stimulatory effect.

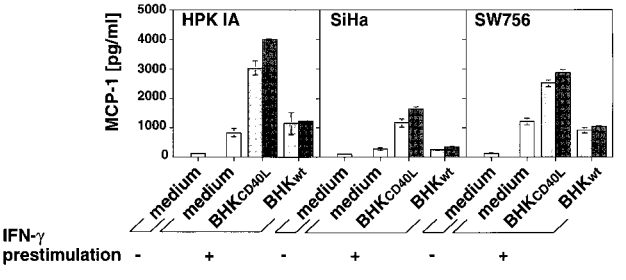

FIGURE 5. CD40 induces MCP-1 in synergism with IFN-γ. Cells were left untreated or were pretreated with 1000 U/ml IFN-γ for 48 h. Then they were stimulated with medium (□), BHK_{CD40L}, or BHK_{wt} cells at 2×10^4 cells/well (▩), or 6×10^4 cells/well (■). Sixteen hours later, the SN were harvested and the MCP-1 levels were determined by ELISA. In all cases, the mean values of duplicate determinations of one representative experiment are shown. In cases in which SDs are not visible, these were below 1% of the mean values.

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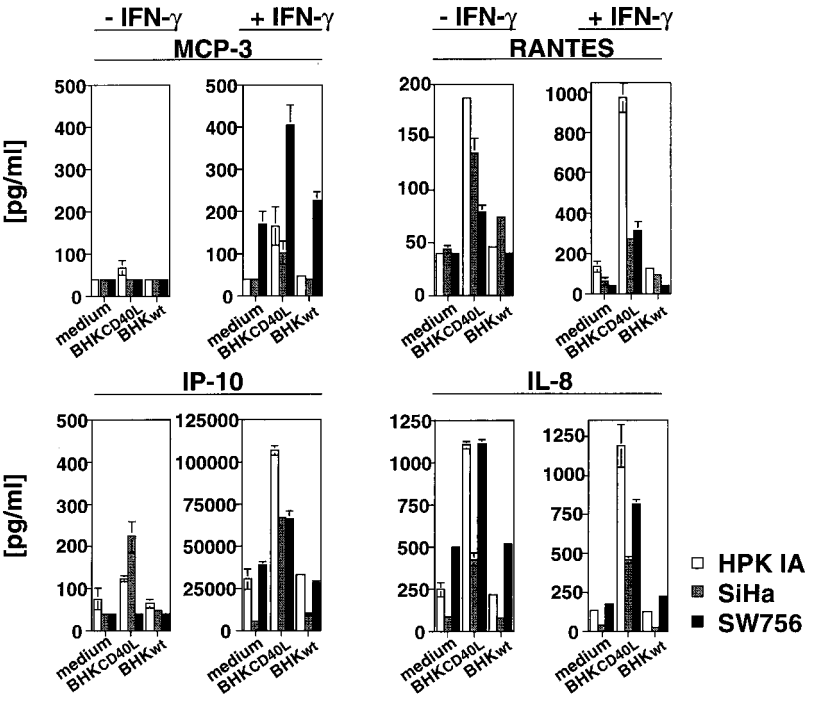

FIGURE 6. CD40 synergizes with IFN-γ to induce MCP-3, RANTES, and IP-10, but not IL-8. HPK IA (□), SiHa (▩), and SW756 (■) were left untreated or were pretreated with 1000 U/ml IFN-γ for 48 h. Then they were stimulated with medium, BHK_{CD40L}, or BHK_{wt} cells at 6×10^4 cells/well. After 16 h, the SN were harvested and the indicated chemokine levels were determined by ELISA. In all cases, the mean values of duplicate determinations of one representative experiment are shown. In cases in which SDs are not visible, these were below 1% of the mean values.

Thus, IL-8 production after CD40L stimulation was reduced in IFN-γ-pretreated cells.

Discussion

Persistent papilloma virus infection of the cervical epithelium may lead to progression from cervical dysplasia to cancer over years or decades. Resolution of the underlying infection depends on complex interactions between the keratinocytes that harbor papilloma viruses and the host immune system. Cell-mediated immunity seems to play a major role in the elimination of the infected keratinocytes (1). Keratinocytes actively participate in the modulation of immune responses, i.e., by secretion of soluble mediators that orchestrate the immune response (46). Thus, chemokines released from keratinocytes may determine whether and which types of immune cells are attracted to the epithelium.

Recently, it has been shown that cervical carcinoma cell lines lack MCP-1 mRNA expression in vitro (32). This chemokine attracts not only monocytes, but also CD4- and CD8-positive memory T cells (47). Studies on MCP-1 in vivo confirmed a low expression in high grade CIN. However, SCC of the uterine cervix showed a mild to strong expression of MCP-1 localized to the cancer cells themselves (31). Interestingly, MCP-1 expression was pronounced at the epithelial-mesenchymal interface and correlated with the inflammatory reaction within the tumor. Those differences in MCP-1 in vivo and in vitro led to the postulate that additional environmental factors were influencing this effect.

In the present study, we demonstrate that the interaction between CD40L and CD40 in synergism with IFN-γ leads to a strong up-regulation of MCP-1 and functionally related cytokines in cervical carcinoma cell lines in vitro, a mechanism that could possibly explain the phenomena observed in vivo.

CD40 was found to be strongly expressed on SCC of the uterine cervix. The ligand for CD40 was detected on a subset of infiltrating mononuclear cells. In vitro, CD40 was strongly expressed on cervical carcinoma cell lines, e.g., SiHa, SW756, and CaSki, corresponding well to the in vivo situation. The only exception were HeLa cells, which were therefore excluded from the study. Foreskin keratinocytes transformed in vitro with HPV16 or 18, i.e., HPK IA, I56, and K51 cells, showed only low CD40 expression levels.

In contrast to the in vivo situation, basal MCP-1 production was low in all cell lines tested in vitro. This low MCP-1 expression could neither be attributed to autocrine production of TGF-β_1, which can suppress MCP-1 production at least in macrophages (43), nor to the lack of the SP-1 transcription factor, which is required for constitutive MCP-1 expression (30). SP-1-binding activity was in fact strong in cervical carcinoma cells (data not

Table I. *Significance of chemokine induction by BHK_{CD40L} compared with BHK_{wt} cells*[a]

Cell Line	Prestimulation	p Values (independent *t* test {CI = 95%})					
		MCP-1	IL-6	MCP-3	RANTES	IL-8	IP-10
HPK IA	w/o IFN-γ	<0.001	ND	0.021	<0.001	<0.001	0.002
	IFN-.γ	<0.001	ND	<0.001	<0.001	<0.001	0.002
SiHa	w/o IFN-γ	<0.001	<0.001	0.980	0.050	0.002	0.001
	IFN-.γ	<0.001	ND	0.025	<0.001	<0.001	0.001
SW756	w/o IFN-γ	<0.001	<0.001	0.793	0.002	<0.001	NC
	IFN-.γ	<0.001	ND	<0.001	<0.001	<0.001	<0.001

[a] w/o, without; ND, not determined; NC, not calculable.

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shown). Therefore, our analyses suggested that in cultured cervical carcinoma cells, the MCP-1 gene might not be repressed in vitro, but might lack stimuli that act on the cells in vivo. As we had detected, both CD40L and CD40 being highly expressed in cervical carcinoma tissue, the consequence of the interaction between both molecules was investigated in vitro. Stimulation of the cervical carcinoma cells (SiHa and SW756) with CD40L induced MCP-1 production. However, when compared with the nontumorigenic cells (HPK IA), MCP-1 production was rather low, despite their much higher CD40 expression levels. Additional experiments revealed that the inducibility of MCP-1 was in a similar range as the inducibility of IL-6 in the carcinoma cells, indicating that there was no selective defect of regulation within the MCP-1 gene itself. As basal IL-6 production was already high in the carcinoma cells, IL-6 was strongly secreted after CD40 ligation.

For efficient gene induction, often two synergistic signals are required. In the case of the IL-6 gene, at least one crucial signal might be constitutively active in cervical carcinoma cells. CD40 activation may provide a second stimulus, probably via NF-κB, which is a regulatory factor for both IL-6 and MCP-1 (30, 48, 49) and is inducible in these cells, as revealed by gel- and supershift analysis. In the case of MCP-1, CD40L provides a stimulatory signal via NF-κB, which, however, seems not to be sufficient to explain the strong up-regulation seen in vivo. As an intriguing hypothesis, T cells might provide a second stimulus not via the same cytokine, but via different effectors. In fact, IFN-γ, another T cell-derived cytokine that induces MCP-1 in endothelial and some epithelial cells (44, 45), also raised MCP-1 production in the cell lines investigated in this study. Stimulation of IFN-γ-primed cells with membrane-bound CD40L led to a strong synergistic response (more than 20-fold), and MCP-1 was produced at nanogram levels. These results suggest that NF-κB activation might be necessary for MCP-1 induction, but other signaling factors, e.g., those induced by IFN-γ, are additionally required to induce MCP-1 production in cervical carcinoma cells.

A similar effect, but even much stronger synergistic induction, was observed for another T cell-attracting chemokine, IP-10, and to a lesser extent for MCP-3 and RANTES. In contrast, neutrophil-attracting IL-8 was induced in both cervical carcinoma cell lines and in vitro transformed cells and reached comparable levels of expression. Furthermore, IL-8 did not respond to IFN-γ pretreatment indicating different regulatory mechanisms of IL-8.

We conclude from our results that production of MCP-1 is not irreversibly repressed in cervical carcinoma cell lines in vitro. The combined signals of CD40L and IFN-γ seem to be adequate stimuli to up-regulate MCP-1 production in these cells. In vivo, both CD40 and CD40L are expressed in close vicinity in cervical SCC. IFN-γ produced by activated infiltrating T cells may sensitize the tumor cells to produce MCP-1 after CD40L contact. This paracrine mechanism may account for the high expression levels of MCP-1 at the mesenchymal-epithelial interface in cervical carcinomas observed in vivo, contributing to the control of disease progression. In cervical carcinomas showing no intense inflammatory reaction, therapeutic application of IFN-γ may be considered as a strong sensitizer for endogenous defense mechanisms via CD40L-CD40-mediated responses.

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