Molecular Pathobiology of Human Cervical High-Grade Lesions: Paracrine STAT3 Activation in Tumor-Instructed Myeloid Cells Drives Local MMP-9 Expression

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Abstract

In many tumors, the switch from precancerous lesions to malignancy critically relies on expression of the matrix-metalloprotease MMP-9, which is predominantly provided by infiltrating inflammatory cells. Our study defines a novel molecular cascade, how human neoplastic cells instruct tumor-associated myeloid cells to produce MMP-9. In biopsies of human papillomavirus–associated precancerous cervical intraepithelial neoplasia (CIN III lesions), we show broad activation of the transcription factor STAT3 and coexpression of MMP-9 in perivascular inflammatory cells. For the first time, we establish a causative link between tumor-mediated paracrine STAT3 activation and MMP-9 production by human tumor-instructed monocytes, whereas NF-κB activation is dispensable for this response. Our data provide evidence that STAT3 does not directly induce MMP-9 but first leads to a strong production of the monocyte chemoattractant protein-1 (CCL2) in the nanogram range. In a second phase, autocrine stimulation of the CCR2 receptor in the tumor-instructed monocytes amplifies MMP-9 expression via intracellular Ca2+ signaling. These findings elucidate a critical mechanism in the molecular pathobiology of cervical carcinogenesis at the switch to malignancy. Particularly in tumors, which are associated with infectious agents, STAT3-driven inflammation may be pivotal to promote carcinogenesis, while at the same time limit NF-κB–dependent immune responses and thus rejection of the infected preneoplastic cells. The molecular cascade defined in this study provides the basis for a rational design of future adjuvant therapies of cervical precancerous lesions. Cancer Res; 71(1); 87–97. ©2011 AACR.

Introduction

There is increasing evidence that neoplastic cells actively shape their microenvironment, which is critical for tumor progression (1). Infiltrating cells create a local milieu characterized by chronic inflammation sustaining proliferation and survival of the neoplastic cells (2–4). The exact molecular mechanisms how human (pre)neoplastic cells instruct the infiltrating immune cells to acquire a protumorigenic inflammatory phenotype are still poorly understood.

The different, well-defined stages of human cervical cancer development are an excellent model to study the interactions between (pre)neoplastic epithelial and infiltrating myelomonocytic cells. Cervical cancer is a consequence of persistent infection with high-risk human papillomaviruses (HPV), predominantly HPV16 and -18 (summarized in ref. 5). Persistence of HPV in the epithelium requires immune escape of the virus, and it has been shown that HPV potently suppresses nuclear factor (NF)-κB–mediated immune responses in epithelial cells (6, 7). Neoplastic progression to cancer takes years or decades and develops from low-grade cervical intraepithelial neoplasia (CIN I) through high-grade lesions, CIN II and CIN III (carcinoma in situ). Thus, cervical carcinogenesis is not a direct consequence of viral infection but requires additional tumor-promoting steps.

Although HPV has “anti-inflammatory” properties, high-grade lesions are infiltrated by myelomonocytic cells (8, 9) and this is strongly associated with cancer progression (10, 11). Thus, inflammation is considered as a major cofactor for human cervical carcinogenesis (12, 13).

Inflammation-associated expression of the matrix-metalloprotease/gelatinase MMP-9 plays a crucial role in tumor development, and the transcription factors NF-κB and activator protein-1 (AP-1) are considered as the mediators of MMP-9 induction in different cell types. Elegant in vivo studies in transgenic mice expressing HPV16 early genes under the keratin 14 promoter (K14-HPV16 mice; ref. 14) have underlined the significance of myeloid cell–derived MMP-9 expression in HPV16-driven epithelial carcinogenesis (15, 16).
Targeting MMP-9 expression in macrophages strongly impaired cervical carcinogenesis in this model (16).

In patients, MMP-9 is expressed in up to 100% of high-grade CIN III lesions and cervical squamous cell carcinomas (17–19) with stromal tumor-infiltrating inflammatory cells, that is, macrophages, being an important source. High MMP-9 expression and activity was correlated with poor prognosis for the patients (18). Yet, how HPV-transformed keratinocytes induce MMP-9 production in infiltrating myeloid cells, when progressing from preinvasive to invasive cancer, is not known. Understanding of the key regulatory events of this paracrine cell communication may lead to the design of novel therapeutic strategies.

In this report, we define a novel molecular cascade of signals between human-transformed epithelial cells and myeloid cells resulting in MMP-9 protein induction.

Our data show that tumor-instructed monocytes require stimuli for macrophage differentiation and activation of the signal transducer and activator of transcription STAT3, whereas NF-kB activation is dispensable for MMP-9 induction.

Materials and Methods

Immunofluorescence and immunohistochemical staining

Cervical paraffin-embedded tissue specimens from 14 patients were taken from the local pathology archive. Written informed consent was given and the protocol was approved by the Ethics Committee, University of Cologne. Histological diagnosis of CIN was ascertained by hematoxylin and eosin staining. High-risk HPV positivity was confirmed by PCR and genotyping.

Immunohistochemistry with anti–MMP-9 mAb 56-2A4 (Merck), anti-CD14 NCL-CD14-223 (Novocastra), and anti-phospho-STAT3 (Tyr705; Cell Signaling Technology) was performed according to IHC World (http://www.ihcworld.com).

For double-immunostaining polyclonal anti-IL-6 antibody (2.5 μg/mL, RS102895; Sigma-Aldrich) was added simultaneously. CCR2b inhibitor (50 μmol/L JSI-124 (Cucurbitacin I), 10 μmol/L STAT inhibitor V Statistic, or 10 μmol/L STAT3 Inhibitor III WP1066 (all from Merck), or neutralizing anti–IL-6 antibody (2.5 μg/mL R&D systems) before stimulation. Anti–M-CSF receptor (M-CSFR) antibody (5 μg/mL, Acris) was added simultaneously. CCR2b inhibitor (50 μg/mL, RS102895; Sigma-Aldrich) blocking CCL2 binding to its receptor (21) or 15 μmol/L intracellular Ca²⁺ chelator BAPTA-AM [1,2-bis-(o-aminophenoxy)-ethane-N,N,N′,N′-tetra-acetic acid, tetraacetoxymethyl ester, Biomol] were added to monocytes after removal of conditioned media. Cells were loaded for 1.5 hours with BAPTA-AM. After treatment with conditioned media and AG490 monocyte viability as determined by propidium iodide staining, and flow cytometry was 89.2% as compared with the respective controls; after JSI-124 treatment viability was 88.2% and after BAPTA-AM treatment 92%. Stimulation experiments were performed 2 to 5 times. Paired t-test was used for statistical analysis.

Gelatin zymography

Cellular supernatants (2 μL) were separated on a 10% SDS-PAGE containing 1 mg/mL gelatin (Sigma-Aldrich) as described previously (22) and documented with ChemiDoc XRS (Bio-Rad Laboratories).
Western blot analysis

Equal volumes (20 μL) of supernatants or equal amounts of protein were analyzed by Western blot using anti–MMP-9 mAb (Merck). Stimulated monocytes were resuspended in sample buffer and analyzed using antibodies directed against phospho-STAT3 (Tyr705; Cell Signaling Technology), STAT3 (Santa Cruz Biotechnology), or actin (Chemicon). Secondary antibodies (Dianova) and ECL reagent (Roche) were used for detection.

Electromobility shift analysis

Nuclear extracts from stimulated monocytes were prepared and NF-κB or AP-1 binding activities were determined in electromobility shift analysis as previously described (23, 24).

TNF-α, CCL2, and MMP-9 ELISAs

TNF-α concentrations were determined with the OptEIA Set (BD Bioscience), CCL2, or MMP-9 with DuoSets (R&D Systems) according to the supplier’s instructions. Detection limits were 7.8, 7.8, or 31.25 pg/mL, respectively.

Flow cytometry

Cells were stained with anti-CCR2 mAb150 (R&D Systems) or respective isotype control mAbs (MOPC-141; Sigma-Aldrich) for 1 hour and secondary fluorescein isothiocyanate–conjugated antibodies (Dianova), fixed in 1% paraformaldehyde, and CCR2 expression was determined by flow cytometry (FACScan, Becton Dickinson).

Results

Inflammatory CD14-positive cells are recruited to human CIN III lesions and strongly express MMP-9

We compared human low- and high-grade CIN lesions for inflammatory cell infiltration. In CIN I lesions, few inflammatory cells were noted adjacent to the epithelium. With increased dysplasia, and most obvious in precancerous CIN III lesions, immune cell infiltration became prominent. In parallel, stromal MMP-9 expression was detected in CIN III lesions but not in the low-grade lesions. This is clearly seen in Figure 1A and B, where CIN I and CIN III coexist in the same biopsy. MMP-9 expression was predominant in the stromal infiltrate of high-grade lesions and exceeded by far the epithelial MMP-9 expression.

To characterize, which inflammatory cells express MMP-9, we costained high-grade lesions with anti-CD14 and anti–MMP-9 antibodies. The perivascular infiltrate was dominated by CD14-positive mononuclear cells. MMP-9 staining was confined to cells within the stroma, but absent in cells within vessels. Overlay of both stainings showed that most perivascular CD14-positive mononuclear cells coexpressed MMP-9 (Fig. 1C).

Paracrine induction of MMP-9 in monocytes cocultured with HPV-transformed cells

To further analyze the underlying mechanism of MMP-9 induction, we cultured peripheral blood–derived CD14-positive monocytes alone or in the presence of HPV-transformed keratinocytes allowing direct cell–cell contact for 3 days. Supernatants were analyzed by sensitive gelatin zymography (Fig. 2A). In cocultures of both cell types, gelatinase activity at 92 kDa representing MMP-9 was strongly upregulated. This was observed for cocultures with patient-derived HPV18- or HPV16-positive tumor cells (SW756, C-4L, HeLa, CaSki), and for keratinocytes transformed with HPV16 in vitro (HPKIA). Depending on the donor, monocytes revealed variable but low basal levels of MMP-9. In supernatants of all HPV-transformed cells cultured alone, MMP-9 gelatinase activity was at the detection limit of gelatin zymography.
Serum-derived pro–MMP-2 in the culture medium was detected throughout the experiments. Levels remained unchanged during coculture.

To investigate the role of direct cell–cell contact, and to define the cellular source of MMP-9 in cocultures, transwell chambers were used. After coculture, MMP-9 production by the 2 cell types was analyzed individually by gelatin zymography (Fig. 2B, top) and Western blot (Fig. 2B, bottom). MMP-9 induction was only observed in the myeloid cells correlating very well with the MMP-9 staining results in clinical biopsy samples. This clearly showed that paracrine MMP-9 induction in the myeloid cells does not require direct cell–cell contact.

To exclude mutual activation of both cell types during coculture, monocytes were stimulated with conditioned media from the different HPV-transformed keratinocytes. A 4-hour exposure of monocytes to each of the conditioned media (Fig. 2C, bottom left) was sufficient to induce the same magnitude of MMP-9 response as the 3 days exposure (data not shown). MMP-9 levels ranged between 45 and 100 ng/mL in 5 different donors as determined by ELISA (data not shown). MMP-9 induction was not observed when monocytes were stimulated with conditioned media from normal genital HPV-negative keratinocytes derived from human foreskin or exocervix (Fig. 2C, bottom right). Serum-free primary keratinocyte medium (KBM-2) was included in the controls. These data show that HPV-transformed but not normal keratinocytes constitutively produce soluble factors inducing MMP-9 in monocytes.

Low-level NF-κB activation in monocytes is not sufficient for MMP-9 induction

To elucidate the signaling pathways involved in paracrine MMP-9 induction in monocytes, the MMP-9 activating AP-1 and NF-κB pathways were analyzed. CD14-positive monocytes were incubated with conditioned media from HPV-transformed keratinocytes for 4 hours. LPS or RPMI medium served as controls. Although LPS strongly activated NF-κB, the conditioned media from the different HPV-transformed keratinocytes generated only weak NF-κB responses in monocytes (Fig. 3A). AP-1 was not activated in monocytes by any of the conditioned media within the 4-hour period (data not shown).

To investigate the impact of paracrine low-level NF-κB activation on MMP-9 induction, we used the NF-κB inhibitor Bay 11-7082. This inhibitor interferes with IκB-α phosphorylation and suppresses CD40-mediated NF-κB activation and MMP-9 induction (25). In control experiments, 10 μM Bay 11-7082 completely prevented LPS-induced NF-κB activation (data not shown) and it suppressed LPS-mediated MMP-9 induction back to levels obtained with plain medium (Fig. 3B, left). Surprisingly, when monocytes were stimulated with
conditioned media from the HPV-transformed keratinocytes. Blocking of NF-κB activation with 10 μmol/L Bay 11-7082 did not interfere with MMP-9 production (Fig. 3B, right). Thus, conditioned supernatants from HPV-transformed keratinocytes led to a strong upregulation of MMP-9 in monocytes, for which NF-κB activation was apparently dispensable. Conditioned media from HPV-transformed keratinocytes also failed to induce detectable levels of TNF-α in monocytes, as measured by ELISA (data not shown). Thus, the weak NF-κB activation observed was neither central to MMP-9 induction nor sufficient to elicit a proinflammatory TNF-α response.

Rapid paracrine STAT3 activation in monocytes primes MMP-9 upregulation

A hint for the cellular signaling pathway mediating MMP-9 induction came from neutralization experiments of 2 factors, M-CSF and IL-6, which are strongly upregulated in cervical carcinogenesis in vivo (26, 27). Blocking of the M-CSFR alone suppressed MMP-9 expression by approximately 44%. A combination of anti-M-CSF and anti-IL-6 antibodies, suppressed MMP-9 production by 73% (P = 0.035; Fig. 4A), whereas anti-IL-6 alone had no significant effect. This synergism was particularly interesting, because M-CSF is central for the differentiation of monocytes into macrophages, and IL-6 is a potent activator of the Jak/STAT3 signaling pathway.

In fact, of the various signaling pathways analyzed, only the STAT3 pathway was uniformly activated by conditioned media of all HPV-transformed cells but not normal human foreskin or exocervical keratinocytes (Fig. 4B; Supplementary Fig. S1). Within 5 to 10 minutes the transcription factor STAT3 was potently phosphorylated at Tyr705 as determined by Western blot analysis. The relevance of STAT3 activation for MMP-9 induction was further investigated with the Jak kinase inhibitor AG490 and the highly specific Jak/STAT3 inhibitor JSI-124 (28). Both inhibitors were individually added to monocytes 30 minutes before and during the 4-hour stimulation period with the conditioned media. Then the inhibitor was removed and monocytes were incubated for another 24 hours with fresh RPMI. MMP-9 induction was strongly suppressed by either inhibitor (Fig. 4C). Similar results were obtained with STAT3 inhibitor III WP1066 and STAT inhibitor V Stattic targeting the STAT3-SH2 domain and preventing its association with upstream kinases (data not shown).

These data clearly show that HPV-transformed keratinocytes rapidly activate STAT3 in monocytes in a paracrine manner, and that STAT3 activation is essential for MMP-9 induction.

STAT3 activation initiates a CCL2/CCR2 amplification loop mediating MMP-9 induction

The link between STAT3 activation and MMP-9 induction in monocytes was unclear. So far, no direct binding of STAT3 to the MMP-9 promoter was described (29). We searched for a STAT3-dependent pathway that ultimately induces MMP-9 expression. Of several potential candidates, we identified the involvement of the STAT3-inducible chemokine CCL2 (30). Blocking its receptor CCR2 with the inhibitor RS102895 completely abrogated MMP-9 induction by conditioned media from all HPV-transformed keratinocytes. Blocking was not only effective, when the inhibitor was present during the whole assay, but it was sufficient to add the inhibitor after removal of the conditioned media (Fig. 5A). This suggested involvement of CCR2 in MMP-9 induction in our coculture model and indicated that CCR2 stimulation in monocytes occurs in an autocrine manner subsequent to stimulation with the conditioned media.
Investigating respective receptor and ligand expression levels revealed that monocytes expressed CCR2, but it was not upregulated after stimulation (Fig. 5B). In contrast, we detected a very strong induction of CCL2 in monocytes after the 4-hour pulse stimulation with conditioned media from the different HPV-transformed keratinocytes (between 10 and 80 ng/mL in monocytes of different donors; Fig. 5C, right). These CCL2 levels were more than 100-fold higher than in the conditioned media of the respective transformed keratinocytes (Fig. 5C, left). Conditioned media from normal genital HPV-negative keratinocytes (NFK-I, NFK-II, or NECK) did not induce CCL2 in monocytes (Fig. 5C, right). As shown in Fig. 5D (left), 10 ng/mL of recombinant CCL2 were sufficient to induce robust MMP-9 production in monocytes.

In monocytes, Ca\textsuperscript{2+} signaling is an early event after CCL2 stimulation (31). To specifically interfere with Ca\textsuperscript{2+} signaling, we applied a widely used cell-permeable Ca\textsuperscript{2+} chelator, BAPTA-AM. This intracellular Ca\textsuperscript{2+} inhibitor strongly suppressed MMP-9 induction (Fig. 5D, right).

To test the link between STAT3 activation and CCL2 induction in pulsed monocytes, we blocked STAT3 activation during the 4-hour stimulation period with conditioned media.

Figure 4. Rapid paracrine STAT3 tyrosine phosphorylation in monocytes is induced by HPV-transformed keratinocytes and primes MMP-9 induction. A, monocytes were stimulated with conditioned media from SW756 cells in the absence or presence of 2.5 µg/mL neutralizing anti–IL-6 or 5 µg/mL neutralizing anti–M-CSFR antibodies or a combination of both for 4 hours. After culture for 24 hours in fresh medium, supernatants were analyzed by MMP-9–specific ELISA. Shown is the percentage of MMP-9 expression from 3 different neutralization experiments. MMP-9 induction by conditioned media from SW756 cells was set to 100%. Statistical analysis was done using a 2-tailed paired t test. B, monocytes were stimulated with plain medium or with conditioned media from SW756, C-4I, HeLa, CaSki, and HPKIA for the indicated time intervals. Whole cell extracts were prepared and analyzed by Western blot using Tyr705-phosphorylation-specific anti-STAT3 (top) or non–phosphorylation-specific antibody (middle). Equal loading was controlled using actin specific mAb (bottom). C, monocytes were preincubated for 30 minutes with or without the Jak/STAT3 inhibitors AG490 (100 µmol/L, top) or JSI-124 (2 µmol/L, bottom) and subsequently pulsed with plain medium or conditioned media from transformed keratinocytes for 4 hours. After culture for 24 hours in fresh medium, supernatants were analyzed by zymography.
Figure 5. Impact of autocrine CCL2 signaling on MMP-9 upregulation in monocytes. A, monocytes were pulsed with plain medium or conditioned media from HPV-transformed cells for 4 hours. Cells were washed and cultivated in fresh medium in the presence or absence of 50 μg/mL CCR2 inhibitor RS102895 for another 24 hours. Supernatants were analyzed by zymography. B, monocytes were stimulated as in A. Cells were harvested and analyzed for surface CCR2 expression by flow cytometry. C, baseline CCL2 production of normal and HPV-transformed human keratinocytes (left) compared with stimulated monocytes from 2 different donors (right). Monocytes were stimulated in individual experiments with plain medium or conditioned media from normal or HPV-transformed keratinocytes for 4 hours. After culture for 24 hours in fresh medium, supernatants were analyzed by CCL2-specific ELISA. D, monocytes were pulsed with 10 ng/mL CCL2 (left) or plain medium or conditioned media from HPV-transformed cells (right) for 4 hours. Cells were washed, loaded with 15 μmol/L BAPTA-AM for 1.5 hours, and cultivated in fresh medium for another 24 hours (right). Supernatants were analyzed by zymography.
CCL2 production in monocytes was strongly suppressed by more than 90% for the Jak/STAT3 inhibitor JSI-124 and from 60% to 94% by AG490 (Table 1). The lower efficiency of AG490 to suppress CCL2 production induced by conditioned media from HPKIA corresponded to its lower ability to suppress MMP-9 induction by these conditioned media.

These experiments show that activation of STAT3 in cocultured monocytes leads to a strong upregulation of the chemokine CCL2 in the myeloid cells, which, in an autocrine manner, stimulates the receptor CCR2 mediating MMP-9 induction.

Extensive phosphotyrosine STAT3 activation in high-grade CIN lesions and coexpression of MMP-9 in infiltrating mononuclear cells

Having identified a novel mechanism, of how tumor cells can instruct monocytes to provide a protumorigenic response, we tested our in vitro results in biopsies of CIN III lesions. As shown in Figure 6, the stroma of the lesions is highly infiltrated by CD14-positive cells (Fig. 6A) and the inflammatory infiltrate stained strongly positive for tyrosine 705–phosphorylated STAT3 (Fig. 6B). This staining pattern was specific for high-grade lesions and not observed in the few infiltrating inflammatory cells seen in CIN I lesions.

Our in vitro data suggest sequential STAT3 activation and MMP-9 induction. We hypothesized that some MMP-9–positive cells might retain staining for activated STAT3. Indeed, immunohistochemical costaining revealed scattered double-positive cells in the inflammatory infiltrate with nuclear-phosphorylated STAT3 and cytoplasmic MMP-9 expression. Typically, double-positive mononuclear cells were located adjacent to vessels, suggesting rapid instruction by microenvironmental cues when egressing from the vasculature (Fig. 6C and D).

Discussion

Our study defines a novel STAT3-dependent molecular cascade of signals in human tumor-associated myeloid cells leading to MMP-9 induction, a matrix-metalloprotease critically contributing to epithelial tumorigenesis.

In human cervical carcinogenesis, high levels of MMP-9 expression are correlated with poor clinical prognosis (18). Stromal myeloid MMP-9 is detected in high-grade lesions and invasive cervical carcinoma (17, 18). So far it was unclear how HPV-transformed keratinocytes instruct human myeloid cells to produce MMP-9.

We identified 2 consecutive phases: first, neoplastic epithelial cells rapidly activate STAT3 in monocytes; then, primed monocytes convert the STAT3 signal via an autocrine CCL2/CCR2 amplification loop and downstream Ca\(^{2+}\) signaling into a strong MMP-9 expression. Our current understanding of the sequence of events leading to myeloid MMP-9 induction is summarized in Supplementary Figure S2.

Several lines of evidence support the pivotal role of STAT3 activation in initiating the downstream cascade of events in human cervical carcinogenesis. We show (i) intense STAT3 activation in inflammatory cells of high-grade CIN III lesions;

![Figure 6. Expression patterns of CD14, tyrosine-phosphorylated STAT3, and MMP-9 in inflammatory CIN III lesions. Serial sections of a CIN III lesion were stained by immunohistochemistry with anti-CD14 (A) or Tyr705-phosphorylation–specific anti-STAT3 (B, all red color), Bars, 50 μm. Double immunohistochemistry of CIN III lesions stained with anti–MMP-9 (red color) and Tyr705-phosphorylation–specific anti-STAT3 antibody (C and D, black color). Bars, 10 μm. Double-positive mononuclear cells were predominantly observed adjacent to vessels.](image-url)
(ii) perivascular localization of phosphotyrosine-STAT3 and MMP-9 double-positive cells strongly suggests that this cascade is activated as soon as infiltrating myeloid cells leave vessels and enter the lesion; (iii) neutralization experiments in vitro showed a critical role of STAT3-activating cytokines for MMP-9 induction in tumor-instructed monocytes; and (iv) pharmacological inhibition of the Jak/STAT3 pathway but not of NF-κB activation abrogates MMP-9 induction in monocytes primed with conditioned media from HPV-transformed keratinocytes. The results shown here give novel mechanistic insight into the role of STAT3 activation in immune cells, which has previously been characterized as a potent inhibitor of antitumor immunity and promoter of tumor angiogenesis (32, 33).

In other inflammation-associated cancers, NF-κB plays a key role in the neoplastic or surrounding inflammatory cells (34, 35). Also, for regulating MMP-9 induction in cervical carcinogenesis one might have expected a major role of the proinflammatory NF-κB or AP-1 pathways. However, HPV rather suppresses acute NF-κB–driven inflammatory responses (6, 7). This may allow escaping immunity at early stages and favors persistence of the virus in the epithelium. Accordingly, classical NF-κB–dependent proinflammatory cytokines, like TNF-α, were not detectable in conditioned media of the different HPV-transformed keratinocytes in this study (data not shown).

Only later, at precancerous stages before malignant tumors are fully established, immune cell infiltration is apparently required for progression. Of note, we and other groups have previously shown that IL-6 and M-CSF are highly upregulated in human cervical carcinogenesis in situ (26, 27). IL-6 has been correlated with a negative prognosis in these patients (36). Cervical cancer cells hardly responded to IL-6, and this suggested a paracrine role for IL-6 (27). In various tumor types, IL-6 links inflammation and carcinogenesis (summarized in ref. 37). Our experiments showed that both, M-CSF and IL-6, synergized in paracrine MMP-9 induction in tumor-instructed monocytes. From these results, it is tempting to speculate that cervical neoplastic cells need to instruct immigrating monocytes to differentiate into macrophages, while at the same time potently activating their STAT3 pathway to upregulate MMP-9 efficiently.

STAT3 does not directly activate the human MMP-9 promoter (29). When searching for a link between STAT3 signaling and MMP-9 induction, we identified an interposed autocrine amplification loop engaging CCL2/CCR2 signaling. The central role of myeloid CCL2/CCR2 signaling was verified at different levels including Jak/STAT3-dependent induction by HPV-transformed but not normal keratinocytes in the nanogram-range, blockade of autocrine CCR2 activation and interference with intracellular Ca²⁺ signaling, a major signaling pathway of CCL2/CCR2 in monocytes (31). The involvement of myeloid CCL2 in our experiments corresponded very well to in vivo studies showing that CCL2 expression is localized predominantly to the inflammatory infiltrate in neoplastic cervical lesions (9, 38). One may speculate that myeloid CCL2 induction may compensate for the failure of HPV-positive keratinocytes to express CCL2 (39).

Thus, upregulation of the chemokine CCL2 and subsequent autocrine Ca²⁺-dependent CCR2 signaling is required to translate the STAT3 signal into a strong MMP-9 response. This observation is in line with previous reports showing that CCL2 is STAT3 inducible (30), upregulated by IL-6 signaling in various cell types (40, 41), and has the potential to induce MMP-9 in myeloid cells (42). In contrast to CCR2-deficient mice, where neutrophils may substitute for protumorigenic functions of monocytes (43), in human cervical cancers absence of CCL2 has been correlated with increased survival (44). The involvement of CCL2 is particularly interesting, because it is not only an immune modulating and tumor-promoting factor but, as a potent chemokine, it may further sustain the inflammatory microenvironment in cervical carcinogenesis (45–48).

Our study provides novel mechanistic insight into the inflammatory host response in human cervical carcinogenesis at the switch from premalignancy to malignancy. First, transformed epithelial cells need to acquire the cytokine set capable to activate STAT3 in bystanding myeloid cells. This may be the most stringent selection part in carcinogenesis. However, when this is achieved, a powerful amplification machinery is initiated in the second phase. Even weak paracrine signals are converted into robust protumorigenic responses. More myeloid cells can be attracted via CCL2 to the precancerous lesion, while at the same time the priming of a tumor rejection response is avoided through STAT3-mediated immunosuppression. Particularly in tumors, which are associated with infectious agents, this type of inflammation, in which NF-κB activation is dispensable, may be pivotal.

Our experiments define a complete, novel cascade of signals between human-transformed epithelial cells and myeloid cells, which eventually create a protumorigenic inflammatory tumor milieu.

In summary, these findings provide the basis for a rational design of future (adjuvant) therapies of precancerous lesions of the uterine cervix and potentially also other tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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