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Short Communication

Differential Expression of the Monocyte Chemoattractant Protein-1 Gene in Human Papillomavirus-16-Infected Squamous Intraepithelial Lesions and Squamous Cell Carcinomas of the Cervix Uteri

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Monocyte chemoattractant protein (MCP)-1 is an important factor involved in the cross-talk between mononuclear cells and human papillomavirus (HPV)-infected cervical epithelia. To prove the experimental model of a negative regulatory loop between the expression of the HPV oncogenes E6/E7 and the MCP-1 gene* in vivo*, we examined HPV-16-infected conization/hysterectomy specimens consisting of 6 low- and 6 high-grade squamous intraepithelial lesions (SILs) and 5 squamous cell carcinomas (SCCs) as well as the adjacent mucosa by isotopic RNA* in situ *hybridization. Langerhans cells and stromal macrophages were identified by the antibodies S-100 and PG-M1. E6/E7 expression was restricted to dysplastic or neoplastic keratinocytes, whereas MCP-1 transcripts were detectable in normal, dysplastic, and neoplastic epithelia, in endothelia, and in stromal macrophages. Langerhans cells were always negative. MCP-1 expression was predominant at the epithelial-mesenchymal junctions and especially intense when the stromal macrophage reaction was increased. Generally, the synchronous expression of E6/E7 and MCP-1 was very rare in SILs (2 of 12 cases). In high-grade SIL, MCP-1 expression was observed in 1 of 6 cases, whereas all lesions strongly hybridized with E6/E7 probes. In contrast, 4 of 5 SCCs re-expressed MCP-1, and 2 cases revealed marked transcriptional activities for both E6/E7 and MCP-1. Although preliminary, our observations lend support to the suggestion that the experimental model of transcriptional regulation and exclusion of either HPV E6/E7 or MCP-1 expression is especially pertinent to high-grade SIL, whereas in most SCCs, other environmental factors may influence this relationship. (Am J Pathol 1996, 149:1469–1476)

Cervical cancer is closely associated with a persistent infection of the epithelium by human papillomaviruses (HPVs), in particular with the high-risk types HPV-16 and-18.[1,2] Experimental studies indicate that much of the transforming potential of HPV-16 and -18 and the ability to stimulate proliferation arise from the biological effects of their E6 and E7 oncoproteins, especially from their capacity to bind and alter the function of the regulatory proteins p53 and the retinoblastoma gene product. Even when integration of the HPV genome into the host cell DNA takes place,

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E6 and E7 genes are selectively retained and often transcribed.[3–5] However, HPV *per se* is not sufficient to permanently transform epithelia and to give rise to invasive cervical cancer. This conclusion is evident from theoretical and clinical considerations and takes into account the long latency period between primary HPV infection and cancer and the fact that numerous women with HPV infection do not develop squamous intraepithelial lesions (SILs), and even among the total number of SILs, only a minor proportion progress to invasive cancer.

It has been proposed that the host immune surveillance plays an important role in growth limiting and regression of HPV-associated lesions of the cervix uteri.[6,7] Indeed, several clinical studies point to cell-mediated rather than humoral immune mechanisms in preventing the development and progress of SIL.[8–10] Activated macrophages are likely candidates of local cellular control and may exert their effects directly via cytotoxic actions against virus-infected or neoplastic cells or indirectly by releasing potent cytokines, eg, the tumor necrosis factor TNF-α. In addition, it becomes more and more evident that the keratinocytes themselves and their growth factors, adhesion molecules, and cytokines are important participants of local immunoregulation.[11–13]

The monocyte chemoattractant protein MCP-1 encoded by the JE gene is one of the molecules involved in the cross-talk between keratinocytes and macrophages. It attracts mononuclear cells to infiltrate into the tissue, triggers them to secrete a number of cytokines with different regulatory effects on the recipient cells,[14,15] and is able to suppress tumor growth.[16] A variety of human tumors can produce chemoattractant factors like MCP-1. An expression of the MCP-1 gene by tumor cells has been demonstrated by Takeshima et al[17] in human gliomas or by Sciacca et al[18] in Kaposi's sarcomas. Recently, Negus et al[19] reported on ovarian cancer cells transcribing the MCP-1 gene and producing the corresponding protein in association with macrophage infiltration.

Interestingly, MCP-1 may be a key protein of inter- and intracellular signaling pathways negatively interfering with HPV oncogene expression and tumorigenicity of cervical epithelia. In HPV-18-infected carcinoma cell lines expressing the HPV oncogenes E6 and E7, Rösl et al[20] demonstrated that a transcription of the MCP-1-encoding gene JE is not detectable. However, the JE gene becomes re-expressed in nonmalignant somatic cell hybrids.

The aim of our study was to analyze the expression of the JE gene and the viral oncogenes E6 and E7 in HPV-16-infected conization/hysterectomy specimens from SILs at different risks of progression and SCCs and to compare the expression with the intensity and distribution of the inflammatory reaction and macrophages in the adjacent stroma.

Materials and Methods

Tissue Samples

This report is based on a study of 12 cervical SILs (6 low grade and 6 high grade) and 5 cervical SCCs, selected after histological review from the files of the Department of Histopathology, Clinic of Gynecology and Obstetrics, University of Hamburg. The tissue material consisted of conization/hysterectomy specimens and had been routinely fixed in 4% buffered formalin. Serial sections of 4 to 6 μm in thickness were cut from the paraffin blocks.

Immunohistochemistry (S-100 and PG-M1)

Deparaffinized tissue sections were washed in phosphate-buffered saline (PBS) and preincubated in 0.5% blocking serum for 20 minutes. The following incubation was performed overnight at 4°C using the rabbit anti-cow S-100 antibody strongly cross-reactive with human S-100 A and B (Dako, Glostrup, Denmark) and the monoclonal mouse anti-human macrophage antibody PG-M1 (Dako), which were diluted in 1% bovine serum albumin (Sigma, Deisenhofen, Germany). A detection followed with a biotinylated secondary antibody (Amersham, Braunschweig, Germany) and streptavidin-alkaline phosphatase conjugate (Gibco-BRL, Eggenstein, Germany) each applied for 1 hour at 37°C. After color development with 0.3 mg/ml nitroblue tetrazolium/0.2 mg/ml 5-chromo-4-chloro-3-indolyl phosphate; Gibco-BRL) for 15 minutes in the dark, the slides were mounted in glycerin gelatin without counterstaining.

Detection of HPV Infection

HPV detection was performed by polymerase chain reaction (PCR) using the consensus primers MY09 and MY11 from the L1 region of the HPV genome[21] as well as HPV-16-,[22] 18-, 6/11-, 31-,[23] and 33[24]-specific primers. To exclude contamination errors, appropriate negative controls were involved in the PCR reactions.

Construction of Ribonucleotide Probes

Total RNA was isolated from tissue samples that had been snap-frozen in liquid nitrogen and stored at −80°C by a standard method using guanidinium isothiocyanate and cesium chloride centrifugation.[25] Non-full-length cDNAs were synthesized by reverse transcriptase (RT)-PCR using specific primers for first-strand synthesis. The primers were derived from previously described sequences.[26,27] For first-strand synthesis of the MCP-1 cDNA, the primers were 5′-CTGCTTGGGGTCAGCACAGA-3′ and 5′-CTTCTGTGCCTGCTGCTCAT-3′.

The 221-bp MCP-1 RT-PCR product was treated with T4-polynucleotide kinase and Klenow fragment of polymerase I in the presence of ATP and all four deoxynucleotides. Then it was inserted into the vector pBluescript-IIKS+ previously restricted with *Sma*I. The obtained plasmid was cloned in *Escherichia coli* DH5-α.[28] Sense and antisense orientations of the cloned insert were determined by plasmid double-strand sequencing. Using the labeled linearized antisense probe in Northern blot hybridization, the expected 0.7-kb transcript could be detected. The primers for the HPV E6/E7 genes contain recognition sequences for the enzymes *Xba*I and *Bam*HI, respectively. For first-strand synthesis, the primers were 5′-GCCGGATCCTTATGGTTTCTGAGAACAGAT-3′ and 5′-CGCTCTAGAATGTTTCAGGACCCACAGGAG-3′.

The 567-bp RT-PCR product specific for E6 and E7 of HPV-16 was obtained from an RNA spliced between nucleotides 226 and 409.[22] This amplification product as well as the vector pBluescript-IIKS+ were restricted by *Xba*I and *Bam*HI, purified, ligated, and transformed into *E. coli* DH5-α.

Generation of Labeled Riboprobes

Radioactively labeled antisense and sense RNA probes were generated by run-off transcription in a solution containing 1 μg of linearized plasmid, either T3 or T7 polymerase, 60 μCi of ^{35}S-labeled uridine triphosphate (specific activity, 1000 Ci/mmol), and 1 mmol/L each of the other three precursors as a cold substrate. This procedure yields RNA with a specific activity of approximately 5×10^6 cpm/μg.

In Situ *Hybridization (ISH)*

RNA-RNA ISH was carried out according to Milani et al.[29] Briefly, deparaffinized sections (5 μm) on 3-aminopropyl-triethoxysilane-coated slides were pretreated with 0.2 mol/L HCl for 20 minutes, digested with pronase (Boehringer, Mannheim, Germany) at 600 μg/ml for 10 minutes at room temperature, postfixed with freshly prepared paraformaldehyde (4%) in PBS for 20 minutes on ice, and acetylated. After dehydration in graded ethanols and drying, hybridizations were performed at 50°C for 16 to 18 hours with 4×10^5 cpm antisense or sense probe in 25 μl of hybridization solution containing 50% formamide, 10% dextran, 10 mmol/L Tris/HCl (pH 7.5), 10 mmol/L sodium phosphate (pH 6.8), 300 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L dithiothreitol, 1 mg/ml yeast tRNA (Boehringer), and 1X Denhardt's solution (Sigma). Slides were subsequently washed in modified hybridization buffer for 4 hours at 50°C, subjected for 20 minutes to RNAse (20 μg/ml) at 37°C followed by washing for 30 minutes at 37°C in 100 mmol/L Tris/HCl (pH 7.4), 500 mmol/L NaCl, 1 mmol/L EDTA and and additional washes in 2X standard saline citrate (SSC) and 0.2X SSC. After dehydration in graded ethanols/600 mmol/L ammonium acetate and drying, slides were coated with Kodak NTB 2 emulsion diluted at equal volumes with 600 mmol/L ammonium acetate. Autoradiography was for 14 to 28 days at 4°C. Slides were counterstained with hematoxylin and eosin (H&E).

Microscopic Semiquantitative Evaluation

Evaluation of conventional histology, immunohistochemistry, and autoradiographic signals (see Table 1) was performed on serial sections by two pathologists independently. Questionable cases were reviewed in conference and discrepancies were resolved by discussion.

Results

All 17 Cases were infected with HPV-16. In 2 cases (case 6, low grade SIL, and case 13, SCC; see Table 1), HPV-18 DNA was additionally detected. Infections with HPV-6/11, -31, and -33 were excluded. The main HPV-16 E6/E7 RT-PCR product corresponded to the previously described transcript E6* (data not shown) obtained from RNA spliced at nucleotides 226 and 409.[22] Therefore, ISH was performed with an E6/E7-specific RNA probe obtained from this cloned non-full-length cDNA.

The results of ISH and immunohistochemistry are summarized in Table 1. The phenotypically normal squamous epithelium did not show any E6/E7 expression. Generally, faint MCP-1 hybridization signals were detectable over squamous epithelia at the epithelial-mesenchymal junction (Figure 1A). In addition, vessel wall cells (Figure 1B), particularly en-

Table 1. *Differential Expression of the Genes E6/E7 and MCP-1 Compared with Intensity of Inflammation and Macrophages in SILs and SCCs*

	E6/E7	MCP-1	Inflammation	Macrophages
Low-grade SILs				
1	−	+	+	+
2	−	−	−	−
3	−	+	−	−
4	+	−	+	+
5	+	+	−	−
6	−	−	−	−
High-grade SILs				
7	+++	−	−	−
8	++	−	+	+
9	+++	−	+	+
10	++	−	+	+
11	+++	++	+++	++
12	+++	−	++	+
SCCs				
13	+++	++	+++	++
14	−	+	++	+
15	+++	−	++	+
16	+++	+++	++	++
17	−	+	+++	+++

The intensity of autoradiographic signals was determined by counting the grains over 100 cells of SILs, SCCs, or adjacent normal squamous epithelium and stroma in representative fields of every section and calculating the mean value.[29] The same procedure was carried out with the corresponding serial control sections (sense probes) to establish the intensity of background signals. After subtraction of background signals, cases were grouped as faint, + (4 to <8 times the number of background grains); moderate, ++ (8 to <12 times the number of background grains); strong, +++ (≥12 times the number of background grains); or absent, − (number of grains corresponds to background). Routine H&E stains and immunohistochemical stains were assessed on an arbitrary scale as to the density of the inflammatory reaction and immunohistochemically labeled cells, respectively. Cases were classified as: inflammation/labeled cells absent (−), sparse (+), moderate (++), or dense (+++).

dothelia, and scattered stromal macrophages were positive for MCP-1. Langerhans cells were always negative.

In low-grade SIL, E6/E7 expression was low or almost undetectable. When present (two of six cases), hybridization signals were restricted to the basal epithelial layers. In contrast, dense autoradiographic signals were observed over all cases of high-grade SILs and three of five SCCs (Figure 1, D and F). Autoradiographic signals in SCC were intense over the most immature peripheral third of the atypical epithelium.

Autoradiographic signals specific for MCP-1 were obtained both in SILs and in SCC. However, the presence and strength of hybridization signals differed strikingly between the groups as shown in Table 1. In low-grade SIL, faint autoradiographic signals for MCP-1 were observed in three of six cases, and three of six cases remained negative as did five of six cases of high-grade SIL. It is notable that the single case of high-grade SIL positive for MCP-1 by ISH (Figure 1C) was unique in the density of its inflammatory reaction and the richness of macrophages therein. In contrast, almost all SCCs (four of five) showed faint to strong autoradiographic signals for MCP-1 (Figure 1E). This positivity for MCP-1 coincided with positivity for E6/E7 in two cases, whereas the other two cases were E6/E7-negative.

As determined by conventional staining and immunohistochemistry, the density of inflammation and the intensity of the macrophage response in the adjacent stroma increased progressively from low-grade SIL to SCC. Cases with marked MCP-1 expression at the epithelial-mesenchymal interface always showed a high density of inflammation and numerous macrophages (Table 1), frequently also invading the epithelium.

Discussion

The majority of cervical SCCs are infected by high-risk HPV types, mainly by HPV-16 and -18. Malignant transformation of the epithelial cells is often accompanied by a drastic increase of the transcription of the viral oncogenes E6 and E7 throughout the proliferating layer. In agreement with previous results,[30,31] we showed that the transcription of the viral oncogenes E6/E7 is suppressed in low-grade SILs although strongly switched on in high-grade SILs and most SCCs. The intracellular control of E6/E7 expression via the interaction of keratinocyte-derived transcription factors with the upstream regulatory region of the virus is already an issue of intensive research.[32–34] In addition, however, the

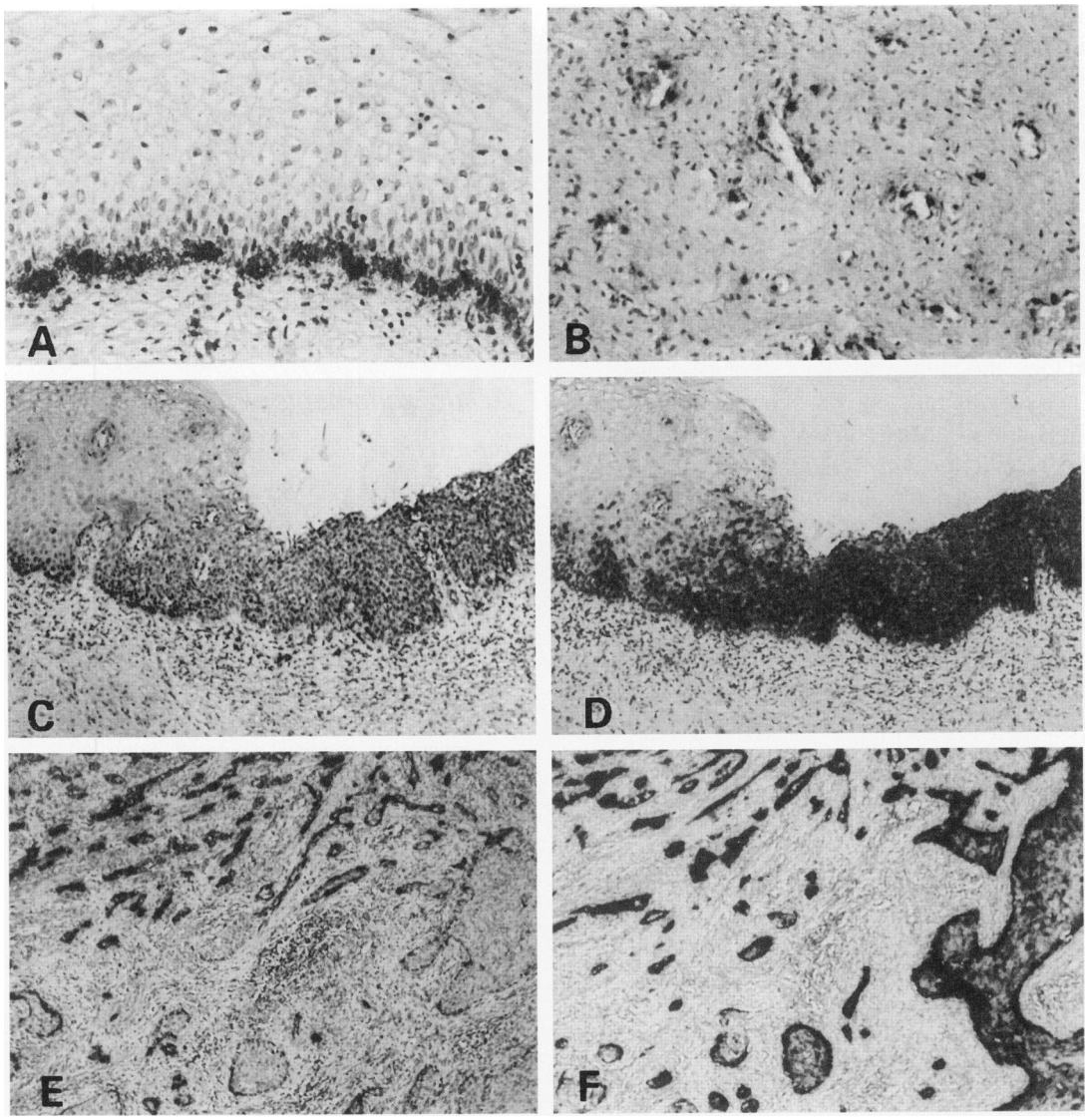

Figure 1. A *and* B: In situ *hybridization with* ^{35}*S-labeled MCP-1 antisense RNA probes. Black grains over basal cells of normal ectocervical squamous epithelium* (A; *magnification,* ×*160) and over vessel wall cells* (B; *magnification,* ×*125*). C *to* F: *In situ hybridization with* ^{35}*S-labeled MCP-1 and E6/E7 antisense RNA probes on serial sections of cervical SIL* (C *and* D) *and SCC* (E *and* F). *There is moderate labeling of MCP-1* (C) *and strong labeling of E6/E7* (D) *over high-grade SIL* (*magnification,* ×*80*) *and intense black autoradiographic signals over neoplastic epithelia of SCC* (*magnification,* ×*50*) *accentuated at the epithelial-mesenchymal interface specific for MCP-1* (E) *and in predominantly diffuse pattern specific for E6/E7* (F). *Exposure time was 21 days. H&E counterstain.*

cellular immune system, especially its monocyte population, is a known source of intra- or intercellular mediators of gene expression and may also participate in down- or up-regulating the HPV oncogene transcription.[35] Using HPV-immortalized cell lines derived from cervical cancer, a number of cytokines have been demonstrated to suppress HPV E6/E7 expression selectively in nonmalignant cells. Such cytokines are, for example, transforming growth factors TGF-β1 and TGF-β2,[36,37] epidermal growth factor EGF,[38] tumor necrosis factor TNF-α, or interferon-γ[20,39] Rösl et al[20] also showed that MCP-1 may be one of the critical molecules linking inter- and intracellular signaling pathways in HPV-associated cervical carcinogenesis. In HeLa cells expressing the HPV-18 oncogenes E6 and E7, the transcription of the MCP-1-encoding gene JE was not detectable. Alterations of the regulatory region or rearrangements of the MCP-1 gene were excluded. Inoculation of such MCP-1-negative HeLa cells into nude mice led to rapidly growing tumors without macrophage infiltration. However, after transfection of HeLa cells with a MCP-1 cDNA carrying expression vector and heterotransplantation into nude mice, a significant growth retardation accompanied by macrophage infiltration was observed.[40]

Interestingly, in the majority of high-grade SIL, although infected by HPV-16 instead of HPV-18, our results from clinical specimens seem to support the *in vitro* observations of Rösl et al.[20] Although all high-grade SILs strongly expressed the viral oncogenes E6/E7, 5 of 6 of them lacked hybridization signals for MCP-1. Low-grade SILs without or with low abundance of E6/E7, on the other hand, displayed an expression of MCP-1 comparable to the normal squamous epithelium in 3 of 6 cases. Generally, the synchronous expression of E6/E7 and MCP-1 was rare in low- and high-grade SILs (2 of 12 cases).

In contrast to SILs, SCCs showed much more heterogeneous patterns. In only one of the three SCCs with strong E6/E7 expression, MCP-1 transcripts were not detectable. Accordingly, a loss of MCP-1 expression does not always apply to invasive carcinomas. Two cases revealed exceptionally high transcriptional activities for both E6/E7 and MCP-1. It is tempting to speculate whether the cellular control mechanisms in culture mimic the microenvironment of preinvasive cancer more closely than in invasive carcinoma with its associated basement membrane disintegration and more intensive interplay with the extracellular matrix and its cellular and humoral components. In this context, it is important to notice the quantitative and qualitative differences of the inflammatory reactions in SILs and SCC. The total density of cellular infiltrates in the adjacent stroma, the proportion of immunohistochemically detected macrophages, and their invasion into epithelia increased continuously from low-grade SILs to SCC. Interestingly, MCP-1 expression was always detected at the epithelial-mesenchymal junctions and was especially intense when the macrophage reaction was increased.

Whether macrophages themselves produce MCP-1 is controversial.[41–43] In our study, vessel wall cells, especially endothelia, and a small proportion of macrophages in the stroma expressed MCP-1, although MCP-1 expression predominated in the cancer cells themselves. Langerhans cells, however, were negative. Recently, Negus et al[19] also demonstrated that tumor cells from primary ovarian cancer as well as from ascites are the major source of MCP-1. At present, however, it is still unknown whether there is any relationship between MCP-1 gene expression and carcinogenesis in general and which factors have an influence on it.

Although previous studies revealed that activated macrophages are able to selectively kill HPV-16-transformed target cells,[44,45] tumor-associated macrophage-derived factors such as epidermal growth factor, platelet-derived growth factor, TGF-β1/TGF-β2, and TNF-α may also promote tumorigenesis.[46] Conceivably, those cytokines are able to influence the regulation of the MCP-1 gene expression and therefore can alter the inverse relationship between MCP-1 production and transcription of viral oncogenes previously demonstrated by Rösl et al[20] for cervical carcinoma cell lines. Thus, it is very important to analyze the role of TNF-α and other cytokines interacting with HPV oncogenes as well as MCP-1 expression and its relationship to carcinogenesis.

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