Differential Regulation of the JE Gene Encoding the Monocyte Chemoattractant Protein (MCP-1) in Cervical Carcinoma Cells and Derived Hybrids

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Malignant human papillomavirus type 18 (HPV18)-positive cervical carcinoma cells can be reverted to a nonmalignant phenotype by generation of somatic cell hybrids with normal human fibroblasts. Although nontumorigenic hybrids, their tumorigenic segregants, and the parental HeLa cells have similar *in vitro* properties, inoculation only of nontumorigenic cells into nude mice results in a selective suppression of HPV18 transcription which precedes cessation of cellular growth. Our present study, aimed at understanding the differential regulation *in vitro* and *in vivo*, shows that the JE gene, encoding the monocyte chemoattractant protein (MCP-1), is expressed only in nontumorigenic hybrids. Although the gene, including its regulatory region, is intact, no JE (MCP-1) mRNA is detected in the tumorigenic segregants and in other malignant HPV-positive cervical carcinoma cell lines. Tests of several monocyte-derived cytokines showed that only tumor necrosis factor alpha strongly induces the JE (MCP-1) gene in nontumorigenic cells and that this is accompanied by a dose-dependent reduction of HPV transcription. The JE (MCP-1) up-regulation occurs within 2 h and does not require *de novo* protein synthesis. The response to tumor necrosis factor alpha seems to be mediated by an NF- κ B-related mechanism, since the induction can be completely abrogated by pretreating the cells with an antioxidant such as pyrrolidine dithiocarbamate. Interestingly, cocultivation of nonmalignant hybrids with monocyte-enriched fractions from human peripheral blood also results in an induction of the JE (MCP-1) gene and a concomitant suppression of HPV18 transcription. Neither effect is observed in malignant cells. These data suggest that JE (MCP-1) may play a pivotal role in the intercellular communication by triggering an intracellular pathway which negatively interferes with viral transcription in HPV-positive nontumorigenic cells.

The etiologic role of specific types of human pathogenic papillomaviruses (HPVs) in a high proportion of cervical cancers and anogenital precancerous neoplasias has been well established (67–69). Approximately 30 distinct HPV genotypes have been isolated from the genital tract, but only a limited number (most notably HPV type 16 [HPV16] but also HPV18, -33, and -52, among others) can be considered high-risk viruses (17). Although the function of the virus-encoded oncogenes E6 and E7 for the maintenance of the malignant phenotype of cervical carcinoma cells *in vitro* and *in vivo* has been proven (16, 60, 61), virus infection per se is not sufficient for cancer development. The small number of infected individuals eventually developing cancer of the cervix, the long latency period between primary infections and cancer emergence, and the monoclonality of the arising tumors clearly indicate the requirement for additional damaging events accumulating in latently HPV-infected host cells (69).

In order to reconcile experimental and epidemiological findings, the hypothesis has been advanced that viral oncogenes are suppressed in nonmalignant proliferating cells by an intracellular surveillance mechanism negatively interfering with viral persistence. Only the functional inactivation or disruption of such a regulatory pathway should result in uncontrolled HPV transcription and tumorigenicity (66).

During the past few years, our group as well as others tried to provide evidence for the existence of a host cell-mediated transcriptional control mechanism interfering with viral transcription and tumorigenicity. It could be demonstrated that HPV16/18 transcription is regulated differently in cells with an altered chromosomal background (10, 45, 46, 53–55). Attempts to identify cellular genes which might be involved in the control of expression of latent HPVs have been partially successful (5, 6, 25, 29, 55).

In situ hybridization studies have shown that heterotransplantation of nonmalignant HPV18-positive HeLa-fibroblast hybrids or of HPV-immortalized human keratinocytes into nude mice leads to a selective suppression of endogenous viral transcription, an effect which is not detectable when RNA expression is monitored under tissue culture conditions (10, 18). Since nontumorigenic hybrids, their tumorigenic segregants, and HeLa cells have comparable growth properties in tissue culture (56), this finding strongly indicates that viral transcription in nonmalignant cells is selectively suppressed *in vivo* by factors probably resulting from intercellular communication with heterologous cell compartments as the consequence of heterotransplantation into an animal (66, 67).

Somatic cell hybrids between cervical carcinoma cells and normal fibroblasts (56) therefore represent a useful model system to examine the abilities of HPV-positive malignant and reverted nonmalignant cells to communicate with components of the immunological network provided after heterografting into nude mice. It is conceivable that such a functional interaction could further trigger an intracellular regulatory

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pathway controlling the proliferation of inoculated cells in vivo. This assumption is supported by recent studies showing that certain macrophage-derived growth-inhibitory cytokines can indeed transcriptionally modulate HPV transcription in nontumorigenic cells (13, 33, 62, 63). But how do cytokines reach their target cells, and what are the activation mechanisms of tumor-associated macrophages?

Since there is little information about regulatory mechanisms involved in the initiation and maintenance of such intercellular cross talk in vivo, we focused our attention on the regulation of the JE gene, which was initially identified as an immediate response gene, inducible by the platelet-derived growth factor (42). The corresponding protein, monocyte chemoattractant protein 1 (MCP-1), represents a small secreted glycoprotein belonging to a superfamily of chemotactic and inflammatory cytokines ("intercrines"; for reviews, see references 37 and 41) which are involved in the chemotactic attraction of monocytes as well as in host defense responses (34). Interestingly, MCP-1 not only stimulates monocyte migration but also activates monocytes to secrete a variety of different cytokines (34, 37), which in turn can exert different biological effects on the recipient cells.

In the present report we show that JE (MCP-1) gene expression is restricted to nontumorigenic HeLa-fibroblast hybrids. Although both the gene and its regulatory region are intact, the corresponding mRNA is detectable neither in malignant segregants of the hybrids nor in other HPV-positive malignant cells. JE (MCP-1) is strongly inducible by certain cytokines (such as tumor necrosis factor alpha [TNF- α]) but solely in nonmalignant HeLa-fibroblast hybrids. In addition, cocultivation with enriched fractions of human mononuclear cells results in a strong induction of JE (MCP-1) only in nontumorigenic cells, and this is paralleled by a selective down-regulation of HPV18 gene expression.

These data point to the importance of MCP-1 in the linkage between inter- and intracellular signalling pathways negatively interfering with HPV expression and potential tumorigenicity under in vivo conditions.

MATERIALS AND METHODS

Cell culture. The following HPV-positive cervical carcinoma cell lines were used in this study: HeLa, SW756, and C4-1, all harboring HPV18 DNA, as well as the somatic cell hybrids between HeLa and normal human fibroblasts (designated 444 for the nontumorigenic hybrid cells and CGL3 for the tumorigenic segregants) (56). SiHa and CaSki cells are positive for HPV16 DNA. C33a is considered an HPV-negative cervical carcinoma cell line, since these cells do not contain any known type of HPV DNA. Both the cervical carcinoma cell lines and the somatic cell hybrids were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% penicillin, and streptomycin.

Cytokines and reagents. The cytokines used were recombinant TNF- α (4×10^7 U/mg; BASF/Knoll, Ludwigshafen, Germany), recombinant interleukin-6 (IL-6) (3×10^7 U/mg; Sandoz, Basel, Switzerland), natural alpha interferon (IFN- α) (3×10^6 U/ml; Bioferon, Rentschler, Laupheim, Germany), recombinant interleukin-1 α/β (IL-1) (2×10^8 U/mg; Hoffmann-LaRoche, Basel, Switzerland), and recombinant IFN- γ (10^7 U/ml; Bioferon). Cycloheximide and pyrrolidine dithiocarbamate (PDTC) were obtained from Sigma Chemicals.

Preparation of monocyte-enriched fractions and cocultivation. Peripheral blood mononuclear cells of healthy donors were isolated from heparinized venous blood by Ficoll-Hypaque (Pharmacia) density centrifugation. Subsequently, 5

$\times 10^7$ peripheral blood mononuclear cells were mixed with 1.5 ml of a 5% sheep erythrocyte suspension, spun for 10 min at 600 rpm, and allowed to incubate for 1 h at 4°C. The cell pellet was resuspended and underlaid with 15 ml of Ficoll-Hypaque. E-rosette-positive (E⁺) and -negative (E⁻) cells were separated by density centrifugation (15 min at 1,500 rpm and 20 min at 2,000 rpm). The E⁻ cells of the interphase were collected, washed twice in RPMI 1640 medium, and resuspended in Dulbecco's modified Eagle's medium in six-well plates at the densities indicated in the figure legends. After 1 h of incubation, nonadherent cells were removed by two washes with a phosphate-balanced salt solution, and the remaining cells were used as a monocyte-enriched fraction (60% CD14⁺, 23% CD2⁺, and 7% CD19⁺, as determined by flow cytometry analysis) for cocultivation with constant amounts (5×10^5) of HPV-positive cells.

PCR synthesis and DNA sequencing. For PCR amplification of the JE (MCP-1) promoter, the following oligonucleotides were used. The 5' primer, 5'-GAATTCAGTTCAATGTTTACACAATC-3', spans a region homologous to nucleotide positions -530 to -505. The 3' primer, 5'-CGAGAGTGCGAGCTTCAGTT-3', is located within the leader region from nucleotide positions +60 to +40 (42, 52). One hundred nanograms of genomic DNA and 50 pmol of each primer were used for 40 cycles of PCR (annealing temperature of 61°C). The authenticity of the amplified fragment was verified by using an internal oligonucleotide (nucleotide positions -276 to -247, 5'-AAAAATAACCCCTCTTAGTTCACATCTGTGG-3') as a hybridization probe. The oligonucleotides were synthesized by the phosphoamidate method with a DNA synthesizer (Applied Biosystems, Inc.) and purified by high-pressure liquid chromatography. The oligonucleotides were labeled by T4 polynucleotide kinase and [γ -³²P]ATP as described previously (48). The PCR products were directly sequenced by using the VENT-polymerase kit (Pharmacia) according to manufacturer's recommendations.

DNA analysis. Genomic DNA was isolated and digested with restriction enzymes by standard procedures (48). The methylation level was monitored by treating the isolated DNA with the isoschizomeric enzymes *MspI* and *HpaII* as described previously (35). *MspI* cleaves independently from the degree of methylation, whereas *HpaII* cannot cleave DNA if the inner cytosine of the *MspI*-*HpaII* recognition site (5'-CCGG-3') is methylated. Digested DNAs were electrophoresed in 1% agarose gels and transferred to GeneScreen Plus membranes (NEN) (48).

Northern (RNA) blot analysis. Total cellular RNA was extracted as described previously (14). Between 2.5 and 5 μ g of RNA was separated in 1% agarose gels in the presence of ethidium bromide under nondenaturing conditions (28) and transferred to GeneScreen Plus membranes (48).

DNA probes. The full-length cDNA of the JE (MCP-1) gene (42) cloned as a 0.74-kb *KpnI* fragment in a pGEM (Promega) vector was obtained from American Type Culture Collection. Unit-length HPV18 DNA cloned in pBR322 (11) was kindly provided by E. Schwarz (Deutsches Krebsforschungszentrum, Heidelberg, Germany). pHF β -A1, harboring an approximately full-length cDNA insert of the fibroblast β -actin gene, was a generous gift from L. Kedes (Medical Center, Palo Alto, Calif.) (23). The human 2'-5' oligo(A) synthetase probe represents an approximately 1.32-kb cDNA insert (9), and IL-6 represents a 1.1-kb insert (65); each is cloned in a pGEM vector. α_2 -Macroglobulin is a 642-bp insert from the human cDNA (8). The probes were labeled with [³²P]dCTP by random priming (20), and hybridizations were performed at 42°C in the presence of 50% formamide.

FIG. 1. JE (MCP-1) expression in cervical carcinoma cells and derived somatic cell hybrids. (A) Lane a, nontumorigenic HeLa-fibroblast hybrids (cell line 444); lane b, tumorigenic segregants of the HeLa-fibroblast hybrids (cell line CGL3); lane c, primary human fibroblasts. (B) Lane a, SiHa cells; lane b, SW756 cells; lane c, C33a cells; lane d, HeLa cells; lane e, primary human fibroblasts. The same filter was rehybridized with a β -actin cDNA probe. The positions of the 28S and 18S rRNAs are indicated.

RESULTS

Expression of the JE (MCP-1) gene is restricted to nontumorigenic HeLa-fibroblast hybrids. To investigate the different regulation of JE (MCP-1) in cervical carcinoma cells and somatic cell hybrids derived therefrom, Northern blot analysis of total RNA was performed. Figure 1 shows abundant expression of the corresponding transcript in primary human fibroblasts (Fig. 1A, lane c, and Fig. 1B, lane e). This cell type was initially used as a normal fusion partner in somatic cell hybridization experiments with tumorigenic, HPV18-positive HeLa cells (56). Although primary human keratinocytes (4) as well as the nontumorigenic HeLa-fibroblast hybrid 444 (Fig. 1A, lane a) exhibit reduced rates of JE (MCP-1) transcription, the mRNA is not expressed at a detectable level in the tumorigenic segregant (CGL3) (Fig. 1A, lane b), in the parental HeLa cells (Fig. 1B, lane d), or in other HPV16- and HPV18-positive cervical cell lines. As demonstrated in Fig. 1B, SiHa (lane a), SW756 (lane b), and C33a (lane c) cells, like CaSki and C4-1 cells (47), lack measurable JE (MCP-1) expression.

To exclude the possibility that the absence of JE (MCP-1) mRNA results from deletion or gross structural rearrangements of the JE gene because of chromosomal instabilities in the cervical carcinoma cell lines, Southern blot and PCR analyses were carried out. Digestion of the genomic DNA with *MspI* and subsequent hybridization with the corresponding cDNA (42) create an approximately 3-kb DNA fragment, which can be discerned in all cell lines (Fig. 2A). The application of the methylation-sensitive enzyme *HpaII*, which is an isoschizomer of *MspI* (35), reveals that there is no correlation between DNA hypermethylation and the absence of gene activity, as described for other transcription units (for a review, see reference 40). Interestingly, JE (MCP-1) expressing nontumorigenic HeLa-fibroblast hybrids (Fig. 2A, lanes b) show a significantly higher degree of DNA methylation than HeLa (lanes c) or CGL3 (lanes d) cells, which do not express the corresponding mRNA (Fig. 1). Moreover, digestion with *EcoRI* or *PstI*, representing either noncutting or singly cutting enzymes (42), also results in an identical cleavage pattern, indicating that there are no rearrangements in the vicinity of the gene (47).

FIG. 2. Genomic analysis of the JE (MCP-1) gene. (A) Southern blot after digestion of the genomic DNA with *MspI*-*HpaII* and hybridization with the JE (MCP-1) cDNA probe. Lanes: a, primary human fibroblasts; b, nontumorigenic HeLa-fibroblast hybrids (cell line 444); c, HeLa cells; d, tumorigenic segregants (cell line CGL3) of the 444 cells; e, C33a cells; f, SW756 cells; g, SiHa cells. The positions of the λ -HindIII marker fragments are indicated on the left of each gel. (B) Ethidium bromide staining of the 591-bp PCR products spanning the JE (MCP-1) regulatory region (42, 52) after hybridization with an internal oligonucleotide. Lanes: a, water control (PCR without addition of genomic DNA); b, primary human fibroblasts; c, nontumorigenic HeLa-fibroblast hybrids; d, HeLa cells; e, tumorigenic HeLa-fibroblast hybrids; f, C33a cells; g, SW756 cells; h, SiHa cells. Lane M represents a mixture of a λ -HindIII and a ϕ -X-HaeIII molecular size marker (23.1, 9.4, 6.5, 4.3, 2.3, and 2.0 kb for λ -HindIII and 1.3, 1.0, 0.87, 0.6, 0.31, and 0.27 kb for ϕ -X-HaeIII).

The presence of a gene detected by cross hybridization with its cDNA does not rule out the possibility that point mutations or other minor genomic alterations within the regulatory region of the JE (MCP-1) gene may be responsible for the lack of transcriptional activity in tumorigenic cervical carcinoma cell lines. To address this question, PCR analysis was performed. By using a specific set of primers flanking the JE promoter, a 591-bp fragment, whose authenticity was verified after hybridization with an internal JE promoter oligonucleotide, can be visualized in ethidium bromide-stained gels (Fig. 2B). Furthermore, direct DNA sequence analysis of the PCR products obtained from primary human fibroblasts (Fig. 2B, lane b) in comparison with amplified DNA from HeLa cells (lane d) reveals that the fragments are identical (42, 52), suggesting that point mutations within the JE (MCP-1) regulatory region cannot account for the absence of gene expression (47). Consequently, the lack of specific transcription

FIG. 3. JE transcription is selectively induced by TNF- α in nontumorigenic HeLa-fibroblast hybrids. HeLa cells, the nontumorigenic HeLa-fibroblast hybrids (cell line 444), and the tumorigenic segregants of the HeLa-fibroblast hybrids (cell line CGL3) (5×10^5 cells each) were incubated for 24 h with different cytokines alone or in combination. Lanes (in each panel): a, untreated control cells; b, TNF- α (250 U/ml); c, TNF- α (250 U/ml) and IFN- γ (100 U/ml); d, IFN- γ (100 U/ml); e, TNF- α (250 U/ml) and IFN- α (100 U/ml); f, IFN- α (100 U/ml). The filters were successively hybridized with JE and β -actin cDNA probes. The positions of the 18S and 28S rRNAs are indicated.

without any detectable alterations at the DNA level indicates that either the genetic background of the nontumorigenic cells (56) or a particular chromatin structure may be important for differential JE (MCP-1) expression (see Discussion).

Cytokines selectively induce JE (MCP-1) in nonmalignant hybrids. JE (MCP-1) is able to activate mononuclear cells, which in turn are induced to secrete a variety of different cytokines (15, 27). There are several lines of evidence that monocyte-derived factors not only mediate host defense responses in vivo (34, 37) but also negatively interfere with HPV transcription and cell proliferation in vitro (13, 33, 62, 63). The restriction of JE (MCP-1) expression to nonmalignant cells is of particular interest in the context of the tumor-suppressing phenotype of HeLa-fibroblast hybrids (cell line 444) after inoculation into nude mice (10). Assuming that under in vivo conditions there is cross talk between nontumorigenic cells and cells of the monocyte/macrophage lineage and vice versa, we tested the influence of several macrophage-derived cytokines on JE (MCP-1) and HPV18 gene regulation in nontumorigenic hybrids (cell line 444), their tumorigenic segregants (cell line CGL3), and parental HeLa cells.

Figure 3 summarizes the influence of TNF- α , IFN- α , and IFN- γ , alone and in combination, on the cell lines. As shown in Fig. 3, lanes b, only TNF- α (like IL-1 [47]) leads to strong induction of the JE (MCP-1) mRNA exclusively in the 444 cell line, whereas IFN- γ (lanes d) or IFN- α (lanes f), like IL-6 (47), fails to enhance the steady-state level of the corresponding gene. There is also no enhancing effect of TNF- α with other cytokines on the augmentation of JE (MCP-1) expression as described recently for primary human keratinocytes (4) or fibroblasts (31). Control hybridizations using the cDNAs of cytokine-inducible reference genes, such as the 2'-5' oligo(A) synthetase for IFN- α and IFN- γ (9), IL-6 for IL-1 (65), and α_2 -macroglobulin for IL-6 (8) treatment confirm that each cytokine was biologically active under our experimental conditions (47).

We next tested the dose response of TNF- α on the HPV18-positive cells by monitoring both JE (MCP-1) and viral expression levels. Incubation of the cells with 50 U of TNF- α per ml was sufficient to induce maximal JE (MCP-1) transcription (Fig. 4, upper panel, lanes b); transcription could not be

increased even after application of higher TNF- α concentrations (lanes c and d). With hybridization of the corresponding total RNA with a viral probe (Fig. 4, lower panel), down-regulation of the HPV18-specific mRNA, which occurs exclusively in the nonmalignant 444 cell line (lanes c and d), can be detected. In contrast to the case with JE (MCP-1) induction

FIG. 4. Dose-dependent down-regulation of HPV18 transcription with TNF- α in nontumorigenic HeLa-fibroblast hybrids. 444, CGL3, and HeLa cells (5×10^5 each) were treated for 24 h with increasing amounts of TNF- α . Lanes b to d, 50, 250, and 500 U of TNF- α per ml, respectively. Lane a, untreated control cells. (Upper panel) Hybridization with the JE cDNA. (Lower panel) Successive hybridization of the same filter with unit-length HPV18 DNA and β -actin cDNA. The positions of the 18S and 28S rRNAs are indicated.

FIG. 5. Time course of JE (MCP-1) induction after TNF- α treatment. Nontumorigenic HeLa-fibroblast hybrid cells (cell line 444) (5×10^5) were incubated with 250 U of TNF- α per ml for 1, 2, 4, and 6 h (lanes b to e, respectively). Lane a, untreated control. The same filter was successively hybridized with JE (MCP-1) cDNA, HPV18, and β -actin probes. The positions of the 18S and 28S rRNAs are indicated.

(see upper panel, lanes b to d, for comparison), the decrease of the HPV18 mRNA steady-state level is dose dependent, since reduction of viral gene expression is seen only after treatment with higher TNF- α concentrations (lanes c and d [250 and 500 U/ml, respectively]). Control hybridizations of the same filter with β -actin (Fig. 4, lower panel) or with *c-myc* (47) demonstrate the selectivity of viral down-regulation, since both cellular genes are not significantly affected and remain constitutively expressed even after incubation with TNF- α concentrations of 500 U/ml (lane d).

Characteristics of TNF- α -mediated JE (MCP-1) induction in nontumorigenic HeLa-fibroblast hybrids. The demonstration that a macrophage-derived factor can specifically stimulate a gene known to be involved in cell-cell communication (37, 41) led to a further characterization of JE (MCP-1) regulation in nonmalignant cells. In an additional set of experiments, the time course of JE (MCP-1) induction in nontumorigenic HeLa-fibroblast hybrids was determined. Incubation with 250 U of TNF- α per ml for different periods of time shows that JE (MCP-1) is rapidly up-regulated and reaches almost its maximal expression level 2 h after cytokine addition (Fig. 5, lane c). No effect on HPV18-specific transcription can be detected during this time period but becomes visible between 4 and 6 h after TNF- α application (Fig. 5, lanes d and e). Consistent with the transient and self-limiting control mechanisms regulating the duration and magnitude of cytokine activity (39), pulse-chase experiments showed that the TNF- α had to be continuously present (24), since the JE (MCP-1) mRNA rapidly declined to the background level 2 h after cytokine removal (47).

We next determined whether JE induction requires ongoing protein synthesis. For this purpose the cells were pretreated for 30, 60, and 120 min with the protein synthesis inhibitor

FIG. 6. TNF- α -mediated JE (MCP-1) induction is independent of de novo protein synthesis. Nontumorigenic HeLa-fibroblast hybrids (5×10^5) were incubated with cycloheximide (5 μ g/ml) alone for 30 min (lane b), 1 h (lane c), and 2 h (lane d) or with cycloheximide in combination with 250 U of TNF- α per ml for an additional 2 h. Lanes e to g, treatment of cells (2 h, 1 h, and 30 min, respectively) with cycloheximide prior to TNF- α addition. Lane h, simultaneous application of TNF- α and cycloheximide for 2 h. Lane i, 2-h incubation with TNF- α alone. Lane a, untreated control cells. The filter was processed for hybridization as described in the legend to Fig. 5. The positions of the 18S and 28S rRNAs are indicated.

cycloheximide (Fig. 6) and were subsequently incubated for an additional 2 h in the presence of TNF- α (lanes e to g, respectively). Northern blot analysis revealed that independently of whether the cells were pretreated (lanes e to g) or whether cycloheximide and TNF- α were added simultaneously (lane h), an approximately equal level of JE (MCP-1) mRNA was accumulated in comparison with that of the cells treated with TNF- α alone (lane i). Interestingly, cycloheximide alone also led to a slight increase of the JE (MCP-1) steady-state level (Fig. 6, lanes c and d). It remains to be seen whether this reflects a stabilization effect on the mRNA (21) or whether the inhibition of synthesis by viral oncogenes E6 and E7 affects JE (MCP-1) transcription.

The rapid induction and the independence from ongoing protein synthesis suggest that a posttranslational process might be involved in JE (MCP-1) activation. Recent studies have shown that the transcription factor NF- κ B is an important regulator of genes involved in inflammatory responses and immunological control processes (1–3). Both TNF- α and IL-1 are cytokines which lead to a rapid dissociation of NF- κ B from its inhibitor in the cytoplasm followed by its translocation into the nucleus (7). The same cytokines also induce reactive oxygen in the cells (36), which represents an important intracellular “second messenger” in the NF- κ B activating process (50). In order to prove an involvement of an NF- κ B pathway in the TNF- α -mediated induction of JE (MCP-1), we used the antioxidant PDTC, which selectively interferes with the release of NF- κ B from its cytoplasmic inhibitor but not with the binding activity of other transcription factors, such as Oct-1, AP-1, or Sp1 (49).

As shown in Fig. 7, TNF- α -mediated JE (MCP-1) induction (lane b) could be completely abrogated in HeLa-fibroblast hybrids when the cells were pretreated with the antioxidant PDTC. A 1-h preincubation period (Fig. 7, lane e) is enough to prevent JE induction completely, whereas simultaneous incubation in the presence of TNF- α for 2 h (lane f) is not sufficient to interfere efficiently with the induction of transcription.

FIG. 7. TNF- α -mediated JE (MCP-1) induction is inhibited by the antioxidant PDTC. Nontumorigenic HeLa-fibroblast hybrid cells (5×10^5) were pretreated with 100 μ M PDTC for 4, 2, and 1 h (lanes c to e, respectively), and subsequently TNF- α (150 U/ml) was added and left for an additional 2 h. Lane a, untreated control cells; lane b, cells incubated with TNF- α (150 U/ml) alone; lane f, simultaneous application of PDTC (100 μ M) and TNF- α (150 U/ml) for 2 h. The filter was hybridized first with the JE cDNA and subsequently with the β -actin cDNA probe.

Control hybridization of the same filter with β -actin shows that PDTC together with TNF- α has no effect on the expression level of a housekeeping gene. Additionally, PDTC chase experiments reveal that 1 h after removal of the antioxidant, the cells again become responsive to TNF- α (47). Although these results do not rule out other activation pathways, the selectivity of PDTC in inhibiting NF- κ B activation suggests a functional role of NF- κ B or of NF- κ B-related factors (26) in the TNF- α -mediated *trans* activation of JE (MCP-1) in non-malignant hybrid cells (see Discussion).

Cocultivation of HPV18-positive cells with enriched fractions of human mononuclear cells. Our data described above demonstrate that monocyte-derived cytokines (such as TNF- α) can selectively induce the JE (MCP-1) gene and that this is accompanied by a reduction of viral gene expression. In order to test the direct effect of mononuclear cells on somatic cell hybrids, cocultivation experiments with enriched monocytes partially purified from peripheral blood of healthy human volunteers were performed (Fig. 8).

The addition of increasing amounts of monocytes to a constant number of nonmalignant HeLa-fibroblast hybrids (cell line 444) or tumorigenic HeLa cells led to a strong induction of JE (MCP-1) expression in 444 cells (Fig. 8, lanes b to f). This up-regulation evidently did not originate from expression of endogenous human monocytes (39), since the corresponding mRNA is only barely detectable after cocultivation with the parental HeLa cells (Fig. 8, lanes b to f) under the same experimental conditions. Concomitant with increasing JE (MCP-1) induction, a significant down-regulation of HPV18 transcription in nontumorigenic HeLa-fibroblast hybrids (cell line 444) can be observed (lanes b to f). As demonstrated by control hybridization, this process cannot be attributed to a dilution of the signal due to the addition of increasing amounts of monocytes, because viral gene expression is not influenced in the tumorigenic segregant CGL3 (47) or in HeLa cells (lanes b to f). Furthermore, reprobing the same membrane with β -actin also confirmed the selectivity in suppressing viral transcription (Fig. 8).

These results suggest that the expression of the JE (MCP-1) gene and its superinducibility play an important role in cell-cell

FIG. 8. JE (MCP-1) induction and HPV18 down-regulation in nontumorigenic HeLa-fibroblast hybrids (cell line 444) after cocultivation with monocyte-enriched fractions from peripheral blood. Increasing amounts of mononuclear cells (lanes b to f [10^6 , 0.25, 0.5, 1, 2.5, and 5, respectively) were cocultivated for 24 h with a constant number (5×10^5) of either HeLa cells or nontumorigenic hybrids (cell line 444). Lanes a, untreated controls. The same filter was subsequently hybridized with JE (MCP-1), HPV18, and β -actin.

communication during cocultivation *in vitro* and possibly also in host defense responses under *in vivo* conditions.

DISCUSSION

Somatic cell hybrids between malignant HPV-positive cervical carcinoma cells and normal human fibroblasts represent a valuable model to study mechanisms of tumor suppression (56) and transcriptional regulation of HPV in cells with different genetic background (5, 10, 46). By use of such an experimental cell system, our present analyses show that the JE (MCP-1) gene (41, 42), which is able to attract and to stimulate monocytes (37, 39), is transcribed exclusively in nonmalignant HeLa-fibroblast hybrids (Fig. 1) and not in their tumorigenic counterparts. The lack of transcriptional activity in malignant cells does not correlate with gross genomic rearrangements or alterations within the regulatory region, since the JE (MCP-1) gene seems to be intact in all malignant cervical carcinoma cell lines tested so far (Fig. 2).

Moreover, because the JE (MCP-1) gene is expressed in primary human keratinocytes (4), this finding raises the question of whether the continuous expression of viral oncogenes such as E6 and E7 may negatively interfere with JE (MCP-1) transcription, an effect which can be compensated for by genetic complementation in somatic cell hybrids made between HeLa and human fibroblasts. In fact, it has been reported that certain oncogenes, such as that for the E1a protein of adenovirus, can suppress JE expression in rodent cells on the level of initiation of transcription (58). Since the E7 protein from high-risk HPV types and adenovirus E1a share conserved regions (38), this structural homology could also account for a similarity of these viral oncogenes in modulating cytokine expression during the multistep process of HPV-linked carcinogenesis.

Although JE (MCP-1) expression is not necessarily correlated with a nonmalignant phenotype (for example, certain glioma cell lines still produce high levels of MCP-1 [64]), the restriction of JE (MCP-1) expression to nonmalignant hybrids is interesting with respect to previous *in vivo* experiments. Inoculation of nontumorigenic HeLa-fibroblast hybrids into

nude mice results in a selective down-regulation of HPV18 transcription (10), while both malignant and nonmalignant cells show continuous viral expression under tissue culture conditions (5, 10, 46). Since cells of the monocyte/macrophage lineage are the main targets of MCP-1 (39), this correlation led to the idea that a paracrine regulatory pathway may play a key role in tumor suppression *in vivo*. In that way, cross talk between nonmalignant cells and components of the immune system would be initiated where monocyte-derived growth factors can exert their immunoregulatory properties on the respective target cells (37, 39).

The examination of various cytokines, such as TNF- α and IFN- γ (Fig. 3), which are known inducers of JE (MCP-1) expression in mononuclear cells (15, 27) but also act in an additive manner in primary human fibroblasts (31) or even synergistically in keratinocytes (4), revealed that TNF- α is the only cytokine which inversely affects JE (MCP-1) and HPV18 expression exclusively in nontumorigenic cells (Fig. 3 and 4). Although HeLa cells *per se* are considered as TNF- α "null" responders (57), the absence of a TNF- α effect in the corresponding tumorigenic counterparts of the somatic cell hybrids (HeLa and CGL3) cannot be attributed to the lack of a functional TNF- α receptor (32) or to a disruption of the TNF- α signal transduction pathway (51), because a combined application of TNF- α and a protein synthesis inhibitor (such as cycloheximide) rapidly induces programmed cell death (47). Apoptosis, which is one characteristic feature of TNF- α mediated cytotoxicity (30), can be seen in all cells independently from the degree of malignancy (47) by the appearance of mononucleosomal repeats (22) within 1 h, using a sensitive end-labeling technique (44).

Since the elevation of the JE (MCP-1) mRNA steady-state level seems to directly correlate with the accumulation of the corresponding protein (42, 43), the strong augmentation of JE (MCP-1) transcription after TNF- α treatment (Fig. 3 and 4) in nonmalignant hybrid cells (cell line 444) could represent a feedback mechanism which in turn enhances monocyte migration as well as the secretion of larger amounts of growth-inhibitory cytokines.

The result that JE (MCP-1) transcription is rapidly increased (Fig. 5) and independent of *de novo* protein synthesis (Fig. 6) is consistent with the requirement that proteins involved in immunological cell-cell communication should be quickly synthesized in order to exert their biological functions in a short period of time. The insensitivity of the TNF- α -mediated JE (MCP-1) induction to cycloheximide treatment also supports the notion that a posttranslational mechanism must be involved in the activation process.

The expression of many genes involved in immune and inflammatory responses (reference 3 and references therein) is regulated by the NF- κ B transcription factor (1, 2). Not only do cytokines, like TNF- α and IL-1, lead to a rapid dissociation of NF- κ B from its cytoplasmic inhibitor protein (I- κ B) and translocation into the nucleus (7), but also reactive oxygen intermediates can act as NF- κ B-inducing stimuli (50, 59). Conversely, the application of an antioxidant such as *N*-acetyl-L-cysteine or PDTTC is a useful tool to inhibit selectively the activity of NF- κ B but not that of other transcription factors (49, 50).

The complete abrogation of TNF- α -mediated JE (MCP-1) induction after pretreatment of nontumorigenic hybrids with PDTTC (Fig. 7) suggests that JE (MCP-1) activation in nonmalignant hybrids is also regulated via such an intracellular pathway. The observation that HeLa cells seem to harbor functionally active NF- κ B (1, 2) but are refractory to JE (MCP-1) induction by TNF- α (Fig. 7) is of particular interest

with respect to the hybrid state of the cells. Since the subunits of NF- κ B (p50 and p65) together with the *c-rel* proto-oncogene belong to a family of transcription factors (1) which, depending on their interaction as homo- or heterodimers, either induce or suppress gene expression (26), it is tempting to speculate that JE (MCP-1) is regulated by specific NF- κ B-like proteins whose association is present only in nontumorigenic cells.

Reports of investigations which support the idea of an involvement of activated macrophages in tumor suppression have been published recently. The introduction of expression vectors encoding the human JE (MCP-1) gene into tumorigenic rodent cells completely prevents tumor formation, an event which is indeed characterized by a pronounced accumulation of monocytes at the sites of injection (43). A similar phenomenon was also observed with murine melanoma cells harboring a transfected JE (MCP-1) gene (12). *In situ* hybridization studies of inoculated nontumorigenic HeLa-fibroblast hybrids with JE (MCP-1)-specific probes in combination with a histological examination and evaluation of the grafts for infiltrated macrophages are necessary to prove whether this prediction is also correct in our model system. Moreover, antisense expression vectors directed against endogenous transcription should reveal whether TNF- α -mediated JE (MCP-1) induction can be neutralized *in vitro* and whether nontumorigenic HeLa-fibroblast hybrids can be converted to a tumorigenic phenotype *in vivo*.

Tissue culture studies which suggest an important role for macrophages are provided by the cocultivation experiments between HPV18-positive cells and enriched monocyte fractions from healthy human donors (Fig. 8). The transcriptional induction of JE (MCP-1) concomitant with a suppression of viral RNA in nontumorigenic cells is in fact reminiscent of HPV transcriptional suppression after heterotransplantation of nonmalignant cells into nude mice (10). This finding is further supported by a similar approach conducted with human foreskin keratinocytes (HPK-Ia) immortalized with HPV16 DNA (18, 19). This particular cell line is nontumorigenic in nude mice during early passage but became spontaneously malignant after long-term propagation in tissue culture (17a). Selective suppression of HPV16 transcription was also observed exclusively in nonmalignant HPK-Ia cells and not in their malignant derivatives (47).

Preliminary experiments suggest that the application of a conditioned medium obtained from cocultivations leads only in nontumorigenic cells to an increase of JE expression, while the steady-state level of the viral transcription is not consistently influenced to such an extent as detected during direct cocultivation. This indicates that JE (MCP-1) and HPV transcription are differently, but not necessarily independently, regulated. Since monocyte-derived cytokines stimulate effects that are self-limiting in duration and magnitude (39), there might be an involvement of a short-lived factor in the down-regulation of HPV transcription which is distinct from cytokines inducing JE (MCP-1) expression. The observation that transcriptional down-regulation of HPV is transient even under cocultivation conditions (incubation of cells for longer than 2 days is accompanied by a reexpression of virus-specific RNA) (47) also supports this assumption. Alternatively, besides cytokine induction, MCP-1 can also elevate monocytic surface adhesion molecule expression (27), suggesting that a direct cell-cell contact leading to higher local concentrations of cytokines might also be relevant in mediation of HPV suppression. The role of TNF- α and other cytokines in conditioned medium from activated monocytes is currently being tested by using monoclonal antibodies abrogating monocyte-derived growth factor JE (MCP-1) induction.

Taken together, the experiments described here support the notion that genes involved in cell-cell communication, such as the JE (MCP-1) gene, play a central role in the linkage between extra- and intracellular pathways negatively interfering with HPV expression and tumorigenicity under in vivo conditions.

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