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Constitutive activation of transcription factor AP-1 in cervical cancer and suppression of human papillomavirus (HPV) transcription and AP-1 activity in HeLa cells by curcumin

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The transcription factor AP-1 plays a central role in the transcriptional regulation of specific types of high-risk human papillomaviruses (HPVs) such as HPV16 and HPV18, which are etiologically associated with the development of cancer of the uterine cervix in women. In our study, we investigated the AP-1 binding activity and the expression pattern of different members of the AP-1 transcription factor family (c-Jun, JunB, JunD, c-Fos, FosB, Fra-1 and Fra-2) in different grades of cervical lesions starting from mild dysplasia to invasive cervical tumors, including normal control tissues, using specific antibodies raised against each of the AP-1 members. Results indicate that though AP-1 showed high binding activity and the majority of its members were highly expressed in tumor tissues, there is a distinct pattern of gradual increase of c-fos and a concomitant decrease of fra-1 expression that perfectly match the progression of cervical lesions. While c-fos is highly expressed in invasive cervical tumor, the expression of fra-1 becomes almost nil or absent, but the reverse is true in both controls and early precancerous lesions. These findings corroborate the results obtained in the cervical cancer cell line, HeLa. Interestingly, despite very low or absent AP-1 binding in normal as well as in premalignant lesions, AP-1 transcription and its binding activity was found to be very high in malignant tissues showing a preferential heterodimerization of c-fos with JunB instead of its canonical dimerization partner c-jun. Both *in vivo* and *in vitro* studies demonstrate that the overexpression of c-fos and downregulation of fra-1 expression as well as a change in the dimerization pattern of the AP-1 complex seem to play a crucial role during progression to malignancy. In a previous study, we demonstrated that a synthetic antioxidant, pyrrolidine dithiocarbamate (PDTC) can selectively downregulate HPV expression in human keratinocytes and cervical cancer cell lines. Since a redox regulatory pathway is involved in the expression of HPV that can be modulated by an antioxidant-induced reconstitution of the AP-1 transcription complex, we have used curcumin (diferuloylmethane), an active component of the perennial herb turmeric, which is a potent antioxidant and is well-known for its antiinflammatory and anticarcinogenic activity, to modulate the transcription of AP-1 and HPV. We demonstrate for the first time that curcumin can selectively downregulate HPV18 transcription as well as the AP-1 binding activity in HeLa cells. Most interestingly, curcumin can reverse the expression dynamics of c-fos and fra-1 in this tumorigenic cell line, mimicking the expression pattern observed in normal controls or precancerous lesions. Observation of curcumin-mediated complete downregulation of AP-1 binding activity and reversal of c-fos/fra-1 transcription to a normal state in tumorigenic HeLa cells represents a novel mechanism that can control transcription of pathogenic HPVs during keratinocyte differentiation and progression of cervical cancer. Our study thus provides a basis for developing a novel therapeutic approach to control pathogenic HPV infection by using potent antioxidative agents, such as curcumin.

Key words: human papillomavirus; AP-1; HeLa; cervical cancer; curcumin; c-fos; fra-1

Cervical cancer is caused by infection of specific types of high-risk human papillomaviruses (HPVs), such as HPV types 16 and 18 (HPV16 and HPV18).[1–3] In India, cancer of the uterine cervix is the major cancer harboring HPV (in almost 98%), and more than 90% of patients with this cancer are specifically infected with HPV16.[4,5] Constitutive expression of 2 early genes, E6 and E7, of high-risk HPVs responsible for tumorigenic transformation[6] is mainly dependent on the availability of host-cell transcription factor, activator protein-1 (AP-1). AP-1 plays a key role during development of cervical cancer,[7] because site-directed mutagenesis of the corresponding AP-1 binding sites within the viral upstream regulatory regions (URR) almost completely abolishes transcription of URR-driven reporter constructs, either under transient transfection conditions[8,9] or in stable differentiation-dependent infection assays in organotypic "raft" cultures.[10,11]

The AP-1 transcription factor that plays a crucial role in the transcriptional regulation of almost all HPV types investigated so far,[8] is formed by either homodimerization of 2 jun proteins (c-jun, JunB, JunD) or heterodimerization of Jun and fos proteins (c-fos, FosB, Fra-1 and Fra-2)[12–14] through the "leucine zipper."[13] The binding site for AP-1 was recognized as the TPA (12-O-tetradecanoyl phorbol 13-acetate) response element (TRE) of several cellular and viral genes, including MTIIa, IL-1, SV-40 and polyoma.[15,16] *In vitro* studies have also shown that Jun/Fos heterodimers are more efficient DNA binding proteins than Jun/Jun homodimers.[13] Since DNA binding is a necessary prerequisite of transactivation, the expression of different proteins of the Jun and Fos family is crucial for the activation of downstream genes regulated by AP-1. In a previous study, we demonstrated a higher binding activity of AP-1 in both HPV-positive and HPV-negative cervical carcinoma cell lines, namely HeLa and C33a, respectively, as well as in HPV16-positive human keratinocyte HPK1a cells.

Since AP-1 is indispensable for efficient epithelial tissue–specific gene expression of HPV[8] and it is also regulated during keratinocytes differentiation,[17] we investigated the binding activity of AP-1 in *in vivo* tissue biopsies from different grades of cervical lesions (low grade squamous intraepithelial lesions [LSIL] and high grade squamous intraepithelial lesions [HSIL]), including invasive cancer and normal controls. We show for the first time that the DNA binding affinity of AP-1, as well as the expression of its constituent members, vary as a function of the severity of cervical lesions. We also demonstrate that it is not the c-jun but mainly the junB that heterodimerizes with c-fos to form the functional AP-1 complex during progression.

In addition, we investigated the transcriptional regulatory role of a potential antioxidative agent curcumin (diferuloylmethane), an active component of the perennial herb turmeric, which is extensively used for imparting specific flavor and yellow color to curry and exhibits antiinflammatory and antitumor activity.[18,19] In a previous study, we had demonstrated that the transactivation and DNA binding affinity of AP-1 can be modulated by alterations of the intracellular redox status if the cells are treated with a potent

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antioxidative agent, pyrrolidine dithiocarbamate (PDTC),[7] which can block not only the transcription of endogenous cellular NF-kB–responsive genes such as the monocyte chemoattractant protein 1 (MCP-1) gene,[20] but also selectively suppressed HPV16 expression. Although PDTC induced elevated binding of the AP-1 transcription factor to its cognate recognition sites within the viral regulatory region, it was revealed that the blockage of HPV transcription was mainly due to antioxidant-induced reconstitution of the AP-1 transcription complex.[7]

Curcumin, which exhibits antiinflammatory and antitumor activity,[19,20] has been shown to suppress activation of transcription factor NF-kB.[21] It has also been shown to inhibit human immunodeficiency virus 1 long terminal repeat (HIV 1 LTR)-directed gene expression and viral replication,[22] but the mechanism(s) of these effects is not yet clearly understood.

In our study, we investigated the effects of curcumin in the HPV18-positive cervical carcinoma cell line HeLa and demonstrate that curcumin is not only a potent inhibitor of AP-1, it selectively suppresses HPV transcription in cervical cancer cells. This antioxidant-induced abolition of HPV oncogene expression, coupled with the downregulation of AP-1 binding activity, represents a novel mechanism that can control pathogenic HPV expression during keratinocyte differentiation and progression of cervical cancer.

Material and methods

Tissue specimens

We collected 100 different grades of cervical tissue specimens comprising 50 invasive cancers, 25 each of LSIL and HSIL. A total of 30 normal cervical tissue samples were obtained from the patients undergoing surgery for gynecological problems other than cervical cancer. The tissue samples from women diagnosed to be at different grades were obtained from the patients attending the cancer clinics of Lok Nayak Hospital, Maulana Azad Medical College, New Delhi. Each biopsy specimen was bisected and one-half was subjected to histopathologic examination and the other one-half was immediately frozen in liquid nitrogen for molecular investigation. Only histopathologically proven samples were considered for the study. Informed consent was obtained from each patient before obtaining the biopsy specimens.

Cell culture

The HPV18-positive cervical carcinoma cell line HeLa was maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin. Curcumin was obtained from Sigma Chemicals (St. Louis, MO) and was freshly dissolved in ethanol and diluted in the medium immediately before use.

DNA extraction and PCR detection of HPV

High molecular-weight genomic DNA was isolated from normal, premalignant and tumor tissue specimens by standard proteinase K digestion and a phenol-chloroform extraction procedure.[5,23] Southern blot hybridization as well as PCR for detection for HPV16 DNA and HPV18 DNA were carried out as described previously,[5,23] using type-specific probes and oligonucleotide primers (HPV16 (1), 5′-AAG GCC AAC TAA ATG TCA C-3′; HPV16 (2), 5′-CTG CTT TTA TAC TAA CCG G-3′; HPV18 (1), 5′-ACC TTA ATG AAA AAC CAC GA-3′; HPV18 (2), 5′-CGT CGT TTA GAG TCG TTC CTG-3′). Initially, all DNA samples were tested for the presence of any HPV type by using a pair of consensus primers located within the conserved L1 open reading frame (ORF) of the HPV genome (MY 11, 5′-GCM CAG GGW CAT AAY AAT GC-3′; MY 09, 5′-CGT CCM ARR GGA WAC TGA TC-3′; where M = A+C, W = A+T, Y = C+T, R = A+G). PCR was performed in a 25 μl reaction mix containing 100 ng DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 125 μM of each dNTP (dATP, dCTP, dGTP and dTTP), 5 pmol of each oligonucleotide primer and 0.5 U Taq DNA polymerase (Perkin-Elmer Biosystems, Foster City, CA, USA). The temperature profile used for amplification constituted an initial denaturation at 95°C for 5 min followed by 35 cycles with denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, which was extended for 4 min in the final cycle. The oligonucleotide primers were synthesized in an automated Applied Biosystems DNA synthesizer (Model 381A; Applied Biosystems, Foster City, CA, USA) using the phosphoramidite method and purified in high pressure liquid chromatography (HPLC).

Extraction of RNA and Northern blotting

Cellular RNA from cervical tissue biopsies as well as from cell cultures was extracted from snap frozen biopsies in liquid nitrogen by the single-step acid guanidium thiocyanate phenol-chloroform extraction method[24] and also by using TRI Reagent (Sigma Chemicals) according to the manufacturer's protocol. The quality and quantity of RNA was estimated by electrophoresis of 2 μl of RNA solution on an ethidium bromide–stained 1% agarose gel in 3-[N-morpholino]propane-sulfonic acid (MOPS) buffer. Presence of at least 2 bands corresponding to 28S and 18S RNA was a good indication of high-quality RNA. Concentration of RNA was also estimated by a dual beam ultraviolet (UV) spectrophotometer at a wavelength of 260 nanometers. Northern blotting was carried out using standard protocols.[7]

Preparation of nuclear extract

Nuclear extracts were prepared by the method of Riol *et al.*,[25] with certain modifications. Frozen tissues were minced and resuspended in ice-cold hypotonic buffer (20 mM HEPES [pH 7.6], 20% [vol/vol] glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 100 mM phenyl methyl sulfonyl fluoride (PMSF), 2 μg/ml leupeptin and 10 μg/ml A-proteinin). After 15 min incubation on ice, lysates were centrifuged at 850*g* for 15 min at 4°C in a microcentrifuge. The supernatant was transferred to a new tube and designated as the cytoplasmic extract. The pellet was washed once with hypotonic buffer and extracted on ice with 2.5 times the pellet size of nuclear extraction buffer (20 mM HEPES [pH 7.6], 25% [vol/vol] glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 100 mM PMSF, 2 μg/ml leupeptin and 10 μg/ml A-proteinin). After 1 hr, the extraction mixture was centrifuged at 18,000*g* at 4°C in a microcentrifuge for 15 min. The resulting supernatant was designated as the nuclear extract. The protein concentration of the extracts was determined by the spectrophotometric method and the extract was stored at –70 °C in a deep freezer or in liquid nitrogen until use. Nuclear extract from cultured HeLa cells were also prepared by this method.

Electrophoretic mobility shift assay

For electrophoretic mobility shift assay (EMSA), the following oligonucleotides were used: an AP-1 consensus sequence 5′-CGCTTGA*TGACTCA*GCCGGAA-3′ (consensus binding sites are underlined and italicized), an Oct-1 consensus oligonucleotide 5′-TGTCGAATGCAAATCACTAGAA-3′ and a Sp-1 consensus sequence 5′-ATTCGATCGGGGCGGGGCGAG-3′. The oligonucleotide primers were synthesized in an Applied Biosystems DNA synthesizer using phosphoramidite chemistry. The above oligonucleotides were annealed and labeled with [γ-^{32}P] ATP (3,000 Ci/mmol; Jonaki, Hyderabad, India) by T4 polynucleotide kinase and gel purified in a 15% polyacrylamide gel.[7]

The binding reaction was performed in a 25 μl reaction volume containing 50% glycerol, 60 mM HEPES (pH 7.9), 20 mM Tris-HCl (pH 7.9), 300 mM KCl, 5 mM EDTA, 5 mM DTT, 100 μg of BSA per milliliter, 2.5 μg of poly (dI-dC) and 10 μg of nuclear extract. After 5 min, 10,000 cpm of the [γ-^{32}P] ATP 5′-end labeled double-stranded oligonucleotide probe was added and the incubation was continued for additional 25 min at room temperature. For monitoring AP-1 composition in supershift assays, 2 μg of polyclonal antibodies (Abs) directed against the Jun/fos family mem-

bers (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were added and the reaction mixture was further incubated for 1 hr at 4°C. The following Abs were used: c-Jun Ab (epitope corresponding to aminoterminal domain of mouse c-Jun p39); JunB Ab (epitope corresponding to carboxy terminal domain of mouse JunB); JunD Ab (epitope corresponding to carboxy terminus of mouse JunD); c-fos Ab (epitope corresponding to a highly conserved domain of c-fos p62 of human origin); FosB Ab (epitope corresponding to amino acids within the central domain of the FosB protein of mouse origin); Fra-1 Ab (epitope corresponding to amino terminus of Fra-1 of rat origin) and Fra-2 (epitope corresponding to carboxy terminus of Fra-2 of human origin). The DNA-protein complexes were resolved on 4.5% nondenaturing polyacrylamide gel (cross-linking ratio, 29:1), dried and exposed overnight to Kodak X-Omat Films (Kodak India Ltd., India).

Western blotting

Nuclear extracts used for band-shift analysis (30 μg of protein per lane) were separated in 10% polyacrylamide gel, electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA), and probed with polyclonal rabbit antibodies of the corresponding family members (see Electrophoretic mobility shift assay). The incubation was carried out overnight in PBS supplemented with 5% skim milk powder, 0.05% Tween 20 (Sigma), and different dilutions of respective Abs. The bands were visualized with an anti-rabbit immunoglobulin G (IgG) antibody conjugated with horseradish peroxidase, using the Luminol reagent detection kit (Santa Cruz Biotechnology).

DNA hybridization probes

pHPV 16 and pHPV 18 represent unit-length HPV16 DNA[1] and HPV18 DNA[2] cloned in pBR322. pHF-A1,[26] harboring an approximately full-length insert of the fibroblast *β-actin* gene, was a generous gift from L. Kedes (Medical Center, Palo Alto, CA, USA). The c-DNA for *c-fos*[27] and *WAF* gene[28] were kindly provided by P. Angel (Deutsches Krebsforschungszentrum, Heidelberg, Germany) and B. Vogelstein (John Hopkins University, Baltimore, MD, USA). The DNA harboring the human homolog for the *fra-1* gene[29] was a generous gift from M. Seiki (Cancer Research Institute, Kanagawa, Japan). All probes were labeled by the random-priming method.[30]

Results

All the cervical specimens ($n = 130$), including normal cervical tissues, were employed for HPV detection by PCR; first by L1 consensus primer, which revealed a total HPV positivity of 10, 49 and 98% in normal, premalignant and malignant cervical tissues, respectively. A total of 46% of the premalignant lesions (LSIL 32%; HSIL 60%) and as high as 94% of the cancer cases were positive for high-risk HPV16 and/or HPV18. The binding activity of AP-1 and the expression profile of its all members, e.g., c-jun, junB, junD, c-fos, fosB, fra-1 and fra-2, were analyzed in all spectra of cervical lesions comprising premalignant (LSIL and HSIL), malignant and normal control tissues. The results showed a gradual increase in the binding activity and expression of AP-1 and its members with the increasing severity of the cervical lesions.

Increased DNA binding activity and expression of AP-1 components changes as a function of severity of cervical lesions

For analysis of the expression and DNA binding activity of the AP-1 family of transcription factors during the progression of cervical cancer, we first performed band-shift assays with the nuclear extracts from tissue biopsy specimens collected from cervical cancer with ^{32}P-labeled probe harboring an AP-1 consensus sequence. The binding was found to be specific, since the retarded complex disappeared after competition with a 100-fold molar excess of a homologous (AP-1), but not with a heterologous, cold probe containing the consensus sequence for the transcription factor Oct-1 (Fig. 1). We then used nuclear extracts from normal and different grades of cervical tissues (LSIL and HSIL) including invasive cervical cancer biopsies and, interestingly, we recorded a

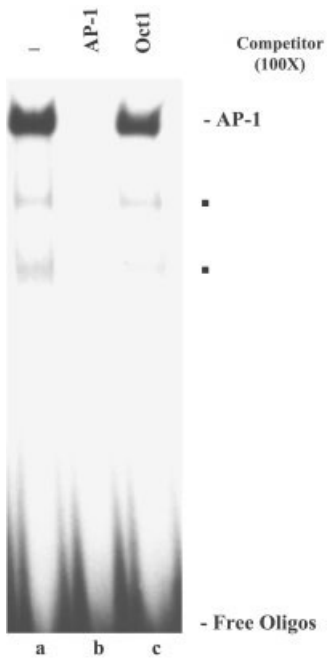

Figure 1 – EMSA using nuclear extracts from invasive cervical cancer tissues, with a ^{32}P-labeled oligonucleotide harboring an AP-1 consensus sequence. Binding specificity was evidenced by preincubation with a 100-fold molar addition of the homologous unlabeled oligonucleotide (Lane b) in comparison with competition experiments using a heterologous consensus sequence of the Oct-1 transcription factor (Lane c). The positions of the specific retarded bands are indicated. The squares mark the unspecific complexes.

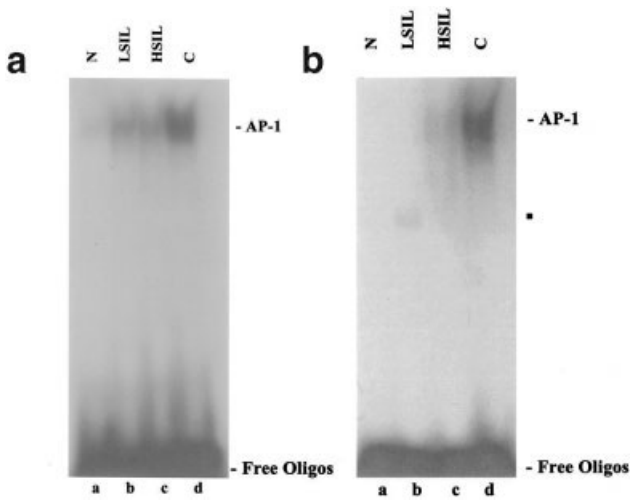

Figure 2 – Gel shift analysis using nuclear extracts from different grades of HPV-positive *(a)* and HPV-negative *(b)* cervical biopsy tissues with ^{32}P-labeled oligonucleotide probe harboring an AP-1 consensus sequence. Increasing AP-1 binding activity was observed as the severity of cervical lesions progressed from normal to invasive cancer (Lanes a–d) in both *(a)* and *(b)*. Lane a, normal controls (N); Lane b, low-grade squamous intraepithelial lesions (LSIL); Lane c, high-grade squamous intraepithelial lesions (HSIL); Lane d, invasive cancer (C). The positions of the specific retarded bands are indicated. The squares mark the unspecific complexes.

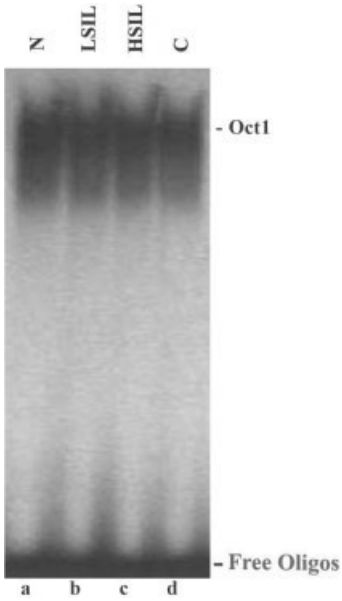

FIGURE 3 – EMSA carried out with labeled oligonucleotides encompassing a consensus sequence of Oct-1 transcription factor showing uniform binding activity in different grades of cervical tissue extracts. Lane a, normal controls (N); Lane b, low-grade squamous intraepithelial lesions (LSIL); Lane c, high-grade squamous intraepithelial lesions (HSIL); Lane d, invasive cancer (C). The positions of the specific retarded bands are indicated.

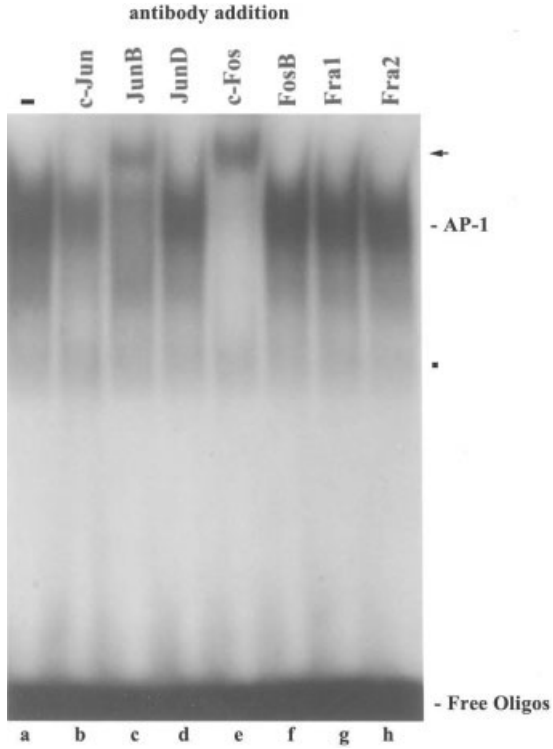

FIGURE 4 – Electromobility supershift analysis using nuclear extracts from invasive cervical tissues with ^{32}P-labeled oligonucleotides harboring an AP-1 consensus sequence showing differential binding activity of AP-1 components. Tumor cell nuclear extracts were incubated with specific antibodies (Abs) recognizing different members of the Fos/Jun family. Lane a, without Ab; Lane b, addition of c-Jun Ab; Lane c, addition of JunB Ab; Lane d, addition of JunD Ab; Lane e, addition of c-Fos Ab; Lane f, FosB Ab addition; Lane g, Fra-1 Ab addition; Lane h, Fra-2 Ab addition. The position of the AP-1 specific complex is indicated. The arrowhead indicates the supershifted bands after antibody addition. The squares mark the unspecific complexes.

gradual increase in the binding activity of AP-1 (Fig. 2*a* and *b*) as the lesion progressed in severity from mild dysplasia (LSIL) to severe lesions (HSIL) to invasive cancer. The specificity of AP-1 binding was reconfirmed in a separate band-shift assay in which we used labeled consensus sequence of Oct-1 as a probe (Fig. 3). While we observed absent or very low DNA binding activity in normal and premalignant tissues, the AP-1 binding activity was found to be highly increased in malignant tissues (Fig. 2*a* and *b*). This trend (normal cervix $<$ LSIL$<$ HSIL $<$ invasive cancer) was observed both in HPV-positive (Fig. 2*a*) as well as HPV-negative (Fig. 2*b*) cervical tissues, showing more of a general phenomenon irrespective of HPV infection. But there was no difference in binding activity between control, LSIL/HSIL or cancer when Oct-1 was used as a probe, which served as a control (Fig. 3).

Alteration in heterodimerization pattern of AP-1 is crucial for progression to malignancy

To understand the role of the elevated binding of AP-1 transcription factor, which is normally indispensable for efficient HPV expression, AP-1 complexes were dissected to their individual components in EMSAs using specific antibodies raised against different members of the AP-1 family, e.g., c-jun, JunB, JunD, c-fos, fosB, fra1 and fra2 (Fig. 4). The supershift analysis revealed a preferential heterodimerization between c-fos and JunB instead of its canonical dimerization partner c-jun. In all malignant tissues analyzed, more than 80% of the supershifted band was formed by c-fos only, while other members of the fos family (fosB, fra1, fra2) showed no or negligible shift after incubation with the respective antibodies. Of the jun family members, JunB was found to be the major dimerization partner for c-fos in the majority of the samples (n = 42/50). However, c-jun and JunD were also found to show a minor shift, in a very few samples.

Expression dynamics of the AP-1 family of proteins in cervical tissues

Western blotting experiments were performed to analyze the level of expression of AP-1 family of proteins during the progression of cervical lesions. Most interestingly, c-jun was found to uniformly exhibit a moderate level of expression from normal controls and premalignant cases to cancer (Fig. 5). In contrast, mainly JunB and partially JunD proteins showed differential expression between normal, premalignant and malignant samples. While a very low or negligible expression of JunB and JunD was observed in normal and premalignant tissues, a very high expression of junB was observed in malignant tissues (see Fig. 5). Among the fos family members, c-fos showed a gradual increase in expression as the severity of lesion increased. While c-fos showed a very low expression in normal samples and a moderate expression in premalignant lesions, the severe dysplastic lesions (HSIL) and tumor tissues showed a very strong expression. Interestingly, a completely opposite trend was observed for Fra-1, which showed a very high expression in normal cervical tissues and it gradually decreased with the increasing severity of the lesion and was almost absent in invasive tumor (Fig. 5). FosB and Fra-2 showed an inconsistent pattern of low to moderate expression in all the types of cervical tissues. Although there were minor differences observed between samples for the expression of the AP-1 family members, in general, they followed the severity-dependent trend of expression. The quality and quantity of expression of the AP-1 family members in normal, premalignant and malignant cervical lesions is presented in Table I.

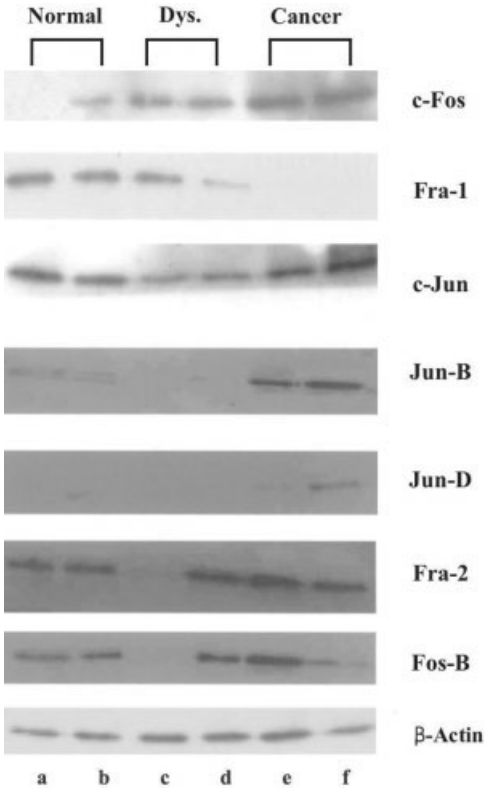

FIGURE 5 – Differential expression pattern of different members of the AP-1 family of proteins in normal, dysplasia (LSIL and HSIL) and cancerous lesions of the uterine cervix. A total of 30 μg protein each from control, dysplasia and cancer cases was separated in a 10% SDS-PAGE minigel. After electrotransfer, the filters were consecutively incubated with different antibodies of AP-1 family (c-fos, Fra-1, c-jun, JunB, JunD, Fra2 and fosB). To confirm equal protein loading, the filters were reincubated with a monoclonal β-actin antibody. Lanes a and b, normal controls; Lane c, low-grade squamous intraepithelial lesions (LSIL); Lane d, high-grade squamous intraepithelial lesions (HSIL); Lanes e and f, invasive cancers. Note that there is a high expression of c-Fos in cancer tissues, while Fra-1 is completely absent.

Transcriptional profile of c-fos and Fra-1 during progression of cervical lesions

The mRNA expression profile of c-fos, Fra-1 and other members of AP-1 was analyzed in different grades of cervical lesions including controls and cancers by Northern blot hybridization, which revealed a complete absence of c-fos expression in normal as well as in premalignant lesions (LSIL), but a moderate to very high expression in severe dysplasia (HSIL) and cervical tumor tissues, respectively (Fig. 6). In contrast, Fra-1 mRNA exhibited a completely reversed pattern of expression; it was very high in control and premalignant tissues, but it gradually decreased as the severity of the lesion increased and it became nil in invasive cancer. The other members of AP-1 exhibited a similar pattern of expression as seen for their proteins (data not shown).

Curcumin can selectively suppress HPV18 transcription in HeLa cells

In a pilot experiment, which was repeated at least 3 times, we incubated the HPV 18-positive cervical carcinoma cells, HeLa, in different concentrations of curcumin (50 μM, 100 μM and 200 μM) for a fixed duration of 1 hr as well as for different time periods (15 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, 6 hr, 12 hr and 24 hr). The results revealed a time- and concentration-dependent decrease in HPV18 expression (Fig. 7). After having analyzed these results, an optimum effective treatment time of 1 hr and a nontoxic dose of 100 μM; curcumin was finalized for further experiments. The cell viability after the curcumin treatment in this concentration was verified by MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay,[31] which showed no cell death, indicating nontoxicity of the dose. Incubation of HeLa cells with 100 μM curcumin for different periods showed that by 2.5 hr of incubation, a slight decline in HPV18-specific mRNA expression was observed and by 4–5 hr the HPV18 expression was completely abolished (Fig. 7). This abolition of HPV transcription was found to be selective and virus-specific and not the consequence of a general transcriptional blockage, since curcumin has no effect on the expression of the endogenous control gene β-actin (Fig. 7). Since the disappearance of HPV18 mRNA follows roughly the same kinetics as observed for HPV16, with the half-life of its mRNA being 2.5 hr,[7] curcumin interference seems to act at the level of initiation of transcription. This is also due to our findings that the disappearance of viral mRNA follows similar kinetics (approximately 2.5 hr) as observed after treatment of HeLa cells with a nonspecific transcription inhibitor, actinomycin D (Fig. 8). The viral mRNA, however, started reappearing by 8–9 hr and regained its normal expression by 12 hr (data not shown) possibly because of complete metabolization of the drug.

Curcumin downregulates the AP-1, but not the SP-1, transcription factor

Incubation of HeLa cells with 100 μM curcumin for different durations indicated time-dependent downregulation of AP-1 binding activity. The AP-1 binding activity started declining after about 2 hr, by which time HPV18 transcription also showed a decline, and became completely abolished by 4–5 hr (Fig. 9). To investigate the specificity of the effect of curcumin on AP-1, we analyzed its effect on other transcription factors such as SP-1. But we could not find any inhibitory effect of curcumin on the SP-1 transcription factor (data not shown).

Curcumin downregulates c-fos, but upregulates fra-1, expression

Most interestingly, when we analyzed the protein expression pattern of c-fos following 100 μM curcumin treatment for different durations (15 min, 30 min, 1 hr, 2 hr, 3 hr and 5 hr), it first showed a decrease in c-fos expression by 2 hr and further decreased gradually in the following hours until it was completely abolished by 4–5 hr. In contrast, fra-1, which was almost nil in untreated HeLa cells, showed its clear appearance by the same time period of 2 hr (when c-fos had started declining) and by 4–5 hr, the fra-1 expression reached its peak (Fig 10). It is very interesting to note that even though the c-fos mRNA level became almost nil after about 2 hr of curcumin treatment (Fig. 7), the c-fos protein remains there up to about 3–4 hr. This may be because the preexisting c-fos protein pool remains for a few hours and is then gradually metabolized, although the transcriptional machinery is blocked by 2 hr of curcumin treatment. Additional experiments were also carried out to detect variation in other AP-1 components after curcumin treatment but no major change in AP-1 composition could be discerned. We also checked the change in composition of the AP-1 binding activity after 100 μM curcumin treatment. Since AP-1 binding activity starts decreasing by 2 hr and becomes nil after around 4–5 hr, we could analyze the AP-1 composition only at 1 hr of curcumin treatment. We found that JunB remains the major component, but involvement of c-fos becomes almost nil (Fig. 11). A very small amount of Fra1 and Fra2 reappears, but the rest of the components showed no change in their involvement.

Curcumin also downregulates p53-responsive gene WAF-1/p21

It has been shown that HPV transcription can be downregulated by p53 and is mediated through the epithelial cell-specific enhancer element within the viral URR.[32] In contrast, our previous study had shown that a synthetic antioxidant, PDTC, however, does not affect p53 expression. To reanalyze this, we examined the expression level of the *WAF-1/p21* gene, representing a known downstream target for altered p53 activity.[28] Thinking that p53

TABLE I – QUALITY AND QUANTITY OF EXPRESSION OF THE AP-1 PROTEINS IN NORMAL, PREMALIGNANT AND MALIGNANT LESIONS OF THE UTERINE CERVIX OF WOMEN[1]

Protein	Normal (n = 30)				Premalignant (n = 50)				Malignant (n = 50)			
	Nil	Weak	Medium	Strong	Nil	Weak	Medium	Strong	Nil	Weak	Medium	Strong
c-Jun	–	8	22	–	–	4	44	2	1	9	38	2
JunB	28	2	–	–	42	8	–	–	–	2	10	38
JunD	30	–	–	–	37	13	–	–	–	4	20	26
c-fos	5	22	3	–	3	30	17	–	–	–	4	46
FosB	0	5	10	15	0	10	20	20	0	28	10	12
Fra-1	1	1	6	22	–	2	18	30	46	4	–	–
Fra-2	0	2	10	18	23	7	18	2	6	20	20	4

[1]Arbitrary level of expression: strong = ++++; medium = ++; weak = +; nil = –.

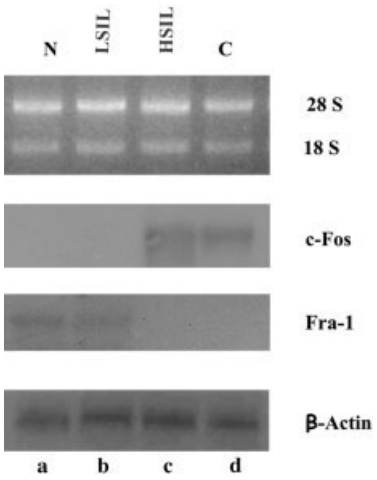

FIGURE 6 – Northern blot analysis showing mRNA expression profile of *c-fos*, *Fra-1* and *β-actin* genes in normal as well as different grades of cervical lesions including cancer. Lane a, normal controls (N); Lane b, low-grade squamous intraepithelial lesions (LSIL); Lane c, high-grade squamous intraepithelial lesions (HSIL); Lane d, invasive cancer (C). To confirm equal RNA loading, the filters were reincubated with *β-actin* probes.

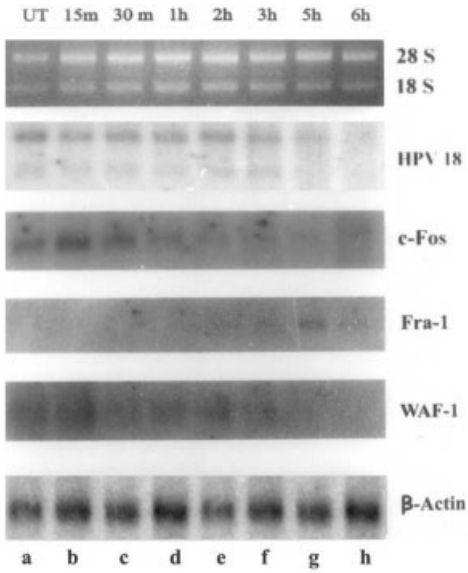

FIGURE 7 – Time-dependent downregulation of HPV 18 transcription in presence of curcumin. Northern blot analysis showing selective downregulation of HPV-18, c-fos and WAF-1 mRNA expression in HeLa cells and upregulation of Fra-1 mRNA expression after 100 μM curcumin treatment at different time intervals. Lane a, untreated cells; Lanes b–h, 15 min, 30 min, 1 hr, 2 hr, 3 hr, 5 hr and 6 hr of incubation in the presence of 100 μM curcumin, respectively. To confirm equal RNA loading, the filters were reincubated with β-actin probes. The positions of the 28S and 18S rRNAs are indicated.

activation is not directly involved in viral gene suppression, we expected to see no change in WAF-1/p21 expression. But to our surprise, we found that as the HPV18 expression gradually decreased, the expression of the cyclin-dependent kinase inhibitor WAF-1/p21 also decreased and became nil after 2 hr of curcumin treatment (Fig. 7). These results indicate that p53 may play a role in HPV downregulation, since unlike PDTC, which has no effect on p53 expression, curcumin downregulates p53 expression. Whether the lack of p53 expression has some later effects needs to be clarified in further experiments.

Discussion

The 2 transforming early genes E6 and E7 of high risk HPV16 and HPV18, possess an intrinsic transactivation capacity on their own homologous promoters,[33,34] but constitutive expression of E6 and E7 in immortalized or malignantly transformed human keratinocytes is mainly dependent on the availability of a defined set of transcription factors, such as AP-1, derived from the infected host cells. Our results show that the AP-1 binding activity is very high in cervical tumor tissues, whereas it is nil or negligible in normal cervical cells. This strengthens the previous hypothesis that the activator protein, AP-1, is not only the major determinant for tissue specificity,[8] but is also absolutely indispensable for efficient HPV oncogene expression.[7] The high binding activity of AP-1 is also observed in several other tumors,[35–39] including cervical tumors that do not contain HPV infection, so its direct relation to HPV is still not very clear.

Assuming that an alteration in the composition of AP-1 either directly or indirectly regulates the transactivation and DNA binding activity, which in turn pushes the normal cells into a premalignant or malignant state, we analyzed the AP-1 binding activity in normal as well as different grades of cervical carcinoma tissues. The most interesting observation was an increased c-fos expression and a decreased Fra-1 expression with the increasing severity of cervical lesions during progression (Fig. 6). A very high expression of c-fos protein was found in malignant cervical tissues, supporting the results of EMSA, which also indicates that the major dimerization partner of active AP-1 complex is c-fos. c-fos as a part of transcription factor AP-1 is known to play a central role in signal transduction by coupling short-term stimulation of the cell to long-term alterations in gene expression.[40] Evidence has also indicated that c-fos alone is capable of transforming the cells *in vitro*.[41] A previous study[42] using nontumorigenic HeLa fibroblast hybrids "444" cells, their tumorigenic segregates "CGL3" and HPV18-positive HeLa cells, showed that c-fos is highly expressed in tumorigenic HeLa and CGL3 cells but completely absent from AP-1 complexes in nontumorigenic 444 cells. Conversely, fra-1 concentration is low or nil in extracts from tumorigenic cells (HeLa and CGL3) and very high in nontumorigenic 444

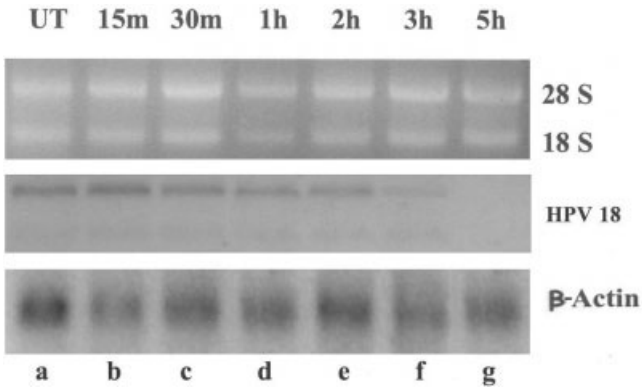

FIGURE 8 – Determination of the half-life of HPV-18 RNA. HeLa cells were treated with 10 μg of actinomycin-D per milliliter and RNA was extracted 15 min, 30 min, 1 hr, 2 hr, 3 hr and 5hr after the addition of the drug. Lane a, untreated cells (UT); Lanes b–g, 15 min, 30 min, 1 hr, 2 hr, 3 hr and 5 hr of incubation in the presence of 100 μM curcumin, respectively. To confirm equal amount of RNA loading, the filters were reincubated with β-actin probes. The positions of the 28S and 18S rRNAs are indicated.

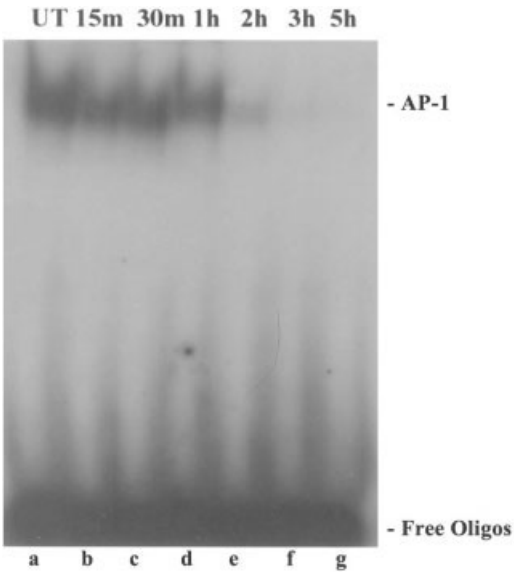

FIGURE 9 – EMSA showing time-dependent downregulation of AP-1 binding activity in HPV-18 positive HeLa cell extracts following the treatment of cells with curcumin for different periods. Lane a, untreated cells; Lanes b–g, 15 min, 30 min, 1 hr, 2 hr, 3 hr and 5 hr of incubation in the presence of 100 μM curcumin, respectively. The positions of the specific retarded bands are indicated. Note that the binding activity of AP-1 diminishes by 2 hr and it completely disappears by 4–5 hr.

cells.[42] Furthermore, ectopic expression of c-fos and reconstitution of sufficient amount of c-fos in 444 cells induced tumorigenicity where fra-1 expression was completely abolished in favor of c-fos, which showed very high expression.[42] We now demonstrate for the first time similar results in *in vivo* tissues from women with precancerous and cancerous lesions and controls and we suggest that c-fos plays a crucial role in the process of tumorigenic transformation of cervical epithelium.

Most interestingly, in a perfect reverse correlation with the upregulated c-fos expression, the fos-related antigen, fra-1, revealed a gradual downregulation with the increasing disease severity and became almost nil in invasive cancer. Similar expression dynamics of c-fos and fra-1 have also been observed in other cancer tissues *in vivo* (Prusty and Das, unpublished results). This is in sharp contrast to what has been reported by other authors in other epithelial cancers.[35–39] The possible explanation for this could be that they have observed overexpression of Fra-1 in carcinoma cell lines, while our findings are mainly in *in vivo* tumor tissue specimens. Our results indicate that fra-1 must have tumor suppressor functions. This is further strengthened by the fact that the fra-1 gene is located in the region of chromosome 11q13,[43] which is known to harbor a tumor suppressor gene and is often structurally deleted or rearranged in cervical cancer cells.[44] It has also been shown that enhanced fra-1 expression can abolish transactivation of c-jun/c-fos complexes on AP-1–responsive genes.[45] Although the overexpression of fra-1 alone has so far failed to cause significant suppression of HPV expression, it has recently been shown to inhibit cell proliferation, induce apoptosis and reduce tumorigenicity of C6 glioma cells.[46] Nevertheless, the contrasting expression dynamics of c-fos and fra-1 appear to play a crucial role during the process of tumorigenic progression of cervical lesions, in which fra-1 appears to play a negative regulatory role during the multistep process of cervical carcinogenesis.

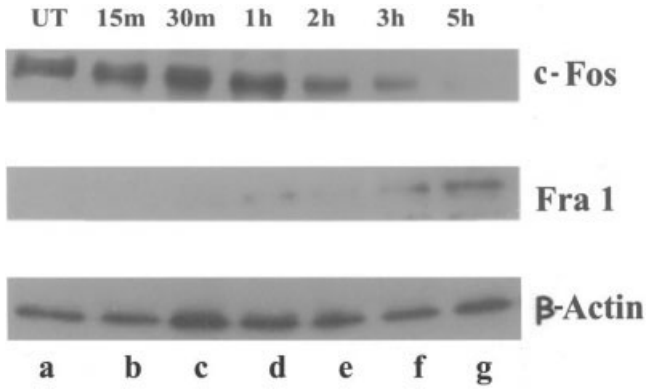

FIGURE 10 – Time-dependent downregulation of c-fos protein expression and upregulation of Fra-1 expression in presence of 100 μM curcumin. HPV-18 positive HeLa cells were treated with 100 μM curcumin for different periods and protein was extracted at each time-point. Separation of 30 μg protein was done in a 10% SDS-PAGE gel. After electrotransfer, the filters were consecutively incubated with c-Fos and Fra-1 antibodies. Lane a, untreated cells; Lanes b–g, 15 min, 30 min, 1 hr, 2 hr, 3 hr and 5 hr of incubation in the presence of 100 μM curcumin, respectively. To confirm equal protein loading, the filters were reincubated with a monoclonal β-actin antibody.

Several different Jun family members, mainly JunB, participated in the increased AP-1 DNA binding activity and were overexpressed in carcinomas. In HeLa cells a 3-fold increase in junB expression has been reported.[47] Interestingly, although c-Jun was consistently expressed in normal, premalignant and malignant tissues *in vivo* in good amount, it was not found to be involved in the dimerization and DNA binding activity, as evidenced in supershift assays (Fig. 4). This confirms that junB constitutes the major dimerization partner of c-fos within the AP-1 complex that participates in tissue-specific transcriptional activation and HPV oncogene expression.[7,48] Studies indicate that c-Jun and JunB function as immediate growth factor response genes and that activation of these 2 genes is required for cell-cycle progression.[49] But c-Jun and junB also have antagonistic functions during oncogenic transformation and cell proliferation.[50,51] It has been shown that junB can largely substitute for c-jun *in vivo*[52] and can inhibit the transforming activities of c-jun.[53] Deregulated junB expression can rescue the jun target genes that are regulated by jun/fos–based sequences. This indicates that the antagonistic effect of junB on jun-mediated transcription is dependent on the physical presence of jun, and supports the previous concept of a transcriptionally

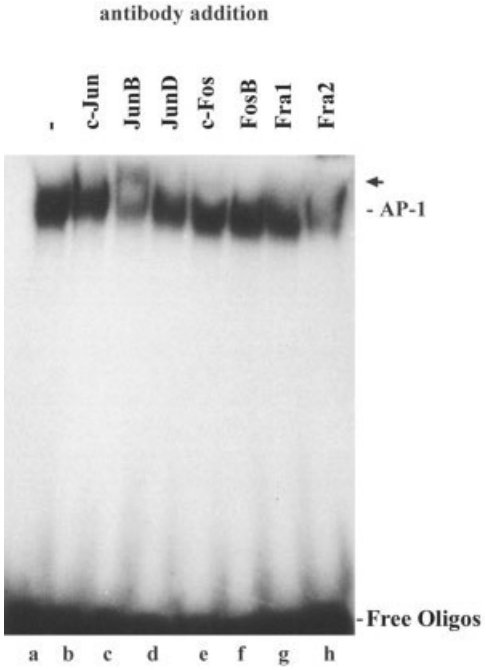

FIGURE 11 – HeLa cells were grown in the presence of 100 μM curcumin for 1 hr. Band supershift assay was done using ^{32}P-labeled oligonucleotides harboring the AP-1 consensus sequence. HeLa cell nuclear extracts were incubated with specific Abs recognizing different members of the Fos/Jun family. Lane a, without Ab; Lane b, addition of c-Jun Ab; Lane c, addition of JunB Ab; Lane d, addition of JunD Ab; Lane e, addition of c-Fos Ab; Lane f, FosB Ab addition; Lane g, Fra-1 Ab addition; Lane h, Fra-2 Ab addition. The position of the AP-1 specific complex is indicated. The arrowhead indicates the supershifted bands after Ab addition.

inactive jun/junB dimer.[53] It can be speculated that in addition to a simple competition with JunB to bind with c-fos, posttranslational modifications of c-Jun may account for the exclusion of c-Jun from AP-1 complex formation.

The AP-1 is also a direct target for the E7 oncogene, which posttranslationally interacts with c-Jun in elevating its transactivation activity.[54] In the absence of c-jun in the AP-1 binding complex, higher junB levels are probably required to restore similar AP-1 binding activity and to ensure similar AP-1–mediated transcriptional activation. A low involvement of JunD in some cases may appear to be paradoxical, since this protein has usually been assigned an antagonistic role in oncogenic transformation. However, recent evidence suggests that this might be an oversimplified explanation, because JunD$^{-/-}$ embryonic fibroblasts display a retarded growth[55] and *menin*, a tumor suppressor gene, when associated with JunD inhibits its transactivation.[56] Although FosB shows inconsistent expression in malignant cervical tissues, its role is still unclear.

The most interesting finding of our study is the selective downregulation of HPV18 transcription in HeLa cells following curcumin treatment. This is the first time it has been demonstrated that expression of HPV can be specifically suppressed by an antioxidant of herbal origin. It follows almost the same kinetics as revealed by treatment with a transcription inhibitor actinomycin D; therefore it is clear that curcumin acts at the level of initiation of transcription, since downregulation of HPV transcription begins by 2.5 hr, which is also estimated to be approximately the half-life of the HPV18-specific mRNA (Figs. 7 and 8).

The observation that curcumin-induced suppression of HPV18 expression is paralleled by concomitant abolition of AP-1 binding to the viral upstream regulatory region (URR) is indicative of the fact that AP-1 is indispensable for efficient viral gene expression. This is in sharp contrast to our earlier observation with a synthetic antioxidant, PDTC, which did suppress HPV expression but increased the binding activity of the AP-1 complex. This has been shown to be caused either by changing the composition of AP-1 and/or by posttranslational modifications.[7] But curcumin appears to follow a different pathway; it does not depend much on the alteration in AP-1 composition, but it acts at the transcriptional level and downregulates the expression of its components. Although the therapeutic potential of curcumin is implicit, the mechanism(s) of curcumin-induced inhibition of AP-1 binding is not yet clearly understood. It is known that DNA binding of the Jun/Fos complex could be modulated by redox regulation of a single conserved cysteine residue located in the DNA binding domain.[57] But it is also possible that curcumin may work in other ways than just as an antioxidant that brings about changes in the redox status of the cell. Whether there exists a curcumin responsive receptor/element or whether curcumin can activate/inhibit some cellular protein(s) or protein kinases that in turn can interact with Jun/Fos binding, is not known. It is possible that curcumin can act through inhibition of JNK, a c-Jun N-terminal kinase needed for AP-1 activation.[58] Another possibility could be that curcumin may directly inhibit formation of the jun-fos DNA complex.[59]

To gain further insight into the effects of curcumin, we performed Northern as well as immunoblot analysis, which revealed a time-dependent downregulation of c-fos expression matching the downregulation of AP-1 activity; but most interestingly, curcumin upregulated fra-1 expression to a level generally observed in normal cervical cells (Fig. 5). This further strengthens the possible role of fra-1 in tumor suppression. Thus the inverse correlation of expression dynamics between c-fos and fra-1, along with the changed heterodimerization pattern between c-fos and jun-B within the AP-1 complex, appears to play a crucial role during development and tumorigenic progression of cervical cancer.

Since p53 is redox-regulated through specific cysteine residues within the DNA binding domain[60] and p53 overexpression alone is sufficient to downregulate HPV16/HPV18 transcription through the epithelial cell–specific enhancer element within the viral URR[61] and since p53-inactivating interaction with HPV16/HPV18 E6 oncoprotein is not an irreversible process, we looked for expression of cyclin-dependent kinase inhibitor WAF-1/p21, which is a known downstream target gene for p53. Interestingly, unlike our previous results with PDTC,[7] which does not affect the steady-state level of p53, or WAF-1, we found downregulation of the p53 target gene *WAF-1* expression, instead of its expected upregulation, indicating a possible role of p53 in downregulating HPV transcription. It remains to be elucidated as to how and by what mechanism(s) the expression of *WAF-1* is downregulated.

Since AP-1 is an indispensable key regulator of epithelial cell–specific transcriptional activity of various HPV types,[7,9,62,63] curcumin-induced abolition of AP-1 binding activity (in which its major binding partner c-fos disappears but fra-1 reappears at a level similar to that in normal controls) is sufficient for selective suppression of HPV oncogene expression. Since curcumin has been shown to have very low or no pharmacological toxicity, our study provides a basis for using potent antioxidative agents such as curcumin for developing novel chemotherapeutic approaches to impair the expression of pathogenic HPVs for control of cervical cancer.

Acknowledgements

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