Short communication

Coincidental expression of E5a and c-jun in human papillomavirus type 6/11-infected condylomata

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Previously, we have shown that E5a can induce expression of the c-jun gene in human papillomavirus (HPV)-11 E5a transformed NIH 3T3 cells and human epidermal keratinocytes. In this study, we investigated the relationship between expression of the E5a gene and c-jun in pathologically confirmed condylomata specimens using mRNA hybridization in situ. The c-jun RNA concentration was significantly higher in condylomata specimens with E5a mRNA expression than in specimens without E5a mRNA expression, or in normal cervical specimens. The cells with c-jun expression were located predominantly in the basal and parabasal cell layers. These layers were also the primary location of E5a-expressing cells. This is the first demonstration of a strong correlation (74%) between expression of the E5a and c-jun genes in condylomata specimens. This correlation might reflect regulation by HPV-11 E5a of c-jun gene expression in condyloma.

Human papillomaviruses (HPVs) are naturally occurring DNA tumour viruses that induce epithelial cell proliferation during the course of productive infection. These viruses are the aetiological agents of condylomata acuminata, laryngeal papilloma, and vulvar and cervical cancer. More than 70 types of HPV have been identified by DNA sequence homology using the molecular hybridization technique (Pfister & Fuchs, 1994). Four of these, HPV-6, HPV-11, HPV-16 and HPV-18 cause primary infections of the genital tract. HPV-6 and -11 are the causal agents of anogenital condyloma acuminata, which infrequently leads to malignant changes (Chen et al., 1993). The predominant viral transcripts of HPV-6 and -11 in genital condylomata potentially encode E1^E4, E5α and E5β proteins (Chow et al., 1987). E5α of HPV-11 can induce foci formation, anchorage independent growth and tumorigenesis in nude mice and NIH 3T3 cells (Chen & Mounts, 1990; Tsao et al., 1994), and anchorage independent growth in C127 cells (Chen & Mounts, 1990) and immortalized human epidermal keratinocytes (Tsao et al., 1994). Previously, we showed that the E5α gene of HPV-11 is required for the initiation of transformation, and that c-Jun may participate in the maintenance of transformation (Chen et al., 1994a, b, 1995).

c-jun is a nuclear oncogene and belongs to the immediate early genes in signal transduction that can regulate cell growth and differentiation (Angel & Karin, 1991). However, the role of c-jun in condylomata has not been explored. In this study, we further investigated the relationship between expression of the E5α gene and c-jun in pathologically confirmed condylomata specimens by mRNA hybridization in situ.

Fifty-four cases of anogenital condyloma acuminata of both males and females in the surgical pathology files of Tri-Service General Hospital from 1991 to 1994 were collected. The prevalence of HPV-6/11 infection in these condylomata was 89% (48 positive cases/54 total tested cases), determined by DNA hybridization in situ, as reported previously (Hsieh et al., 1994). Each tissue block was sectioned at 3 to 4 μm and stained with haematoxylin–eosin. Additional unstained serial sections were mounted on 3-aminopropyltriethoxysaline coated glass microscope slides (Wilson et al., 1992) for analyses by mRNA hybridization in situ in this work. Ten cases of normal anogenital squamous epithelia were randomly selected from surgical specimens and processed by the procedures described above.

Construction of message-specific probes was done with the following methods. Plasmid pSL13, which contains an E5α DNA fragment in pBluescript, was linearized with EcoRI or BamHI in the multiple cloning site distal to the T7 or T3 promoter respectively. Transcription driven by the T7 promoter of plasmid
Fig. 1. For legend see opposite.

Fig. 2. For legend see opposite.
relations for RNA:RNA hybrids have been described to eliminate non-specific retention of probes. 

$T_m$ calculations for each of the probes was performed on serial sections of conventional formalin-fixed and paraffin-embedded biopsies of condylomata. High stringency washes, were carried out in 0.1 x SSC (0.15 M NaCl, 0.015 M-sodium citrate) at approximately $T_m -5 ^\circ C$ to eliminate non-specific retention of probes. $T_m$ calculations for RNA:RNA hybrids have been described (Bodkin & Knudson, 1985). According to our previous observations, there is no cross-hybridization among c-jun, junB and junD under highly stringent conditions (Chen et al., 1995). Slides were then dipped in liquefied Kodak NTB-2 radiographic emulsion, exposed at 4 °C for 1 week, and photographically developed in Kodak D19. The sections were counterstained with haematoxylin–eosin, and photographed.

To determine the relationship between expression of E5a mRNA and c-jun mRNA, in situ hybridization with both RNA probes was carried out on serial sections of condylomata from 31 female condylomata cases (27 cases were HPV-6/11 positive), 23 male cases (21 cases were HPV-6/11 positive), and 10 samples of normal cervical tissues (without HPV-6/11 infections). Of the condylomata cases 91% expressed E5a mRNA and 9% did not, as shown in Table 1. However, of the 49 cases of E5a mRNA positive condylomata, 48 were HPV-6/11 positive; only one case of female condylomata was negative for HPV-6/11 infection. For this HPV-6/11 negative case, amplification of the undetectable viral genome by transcription may have resulted in detection of viral mRNA by in situ hybridization. Of the 54 condylomata specimens tested, 40 expressed E5a mRNA and had higher expression of c-jun (Table 1; Fig. 1a, d) than was found in specimens without E5a mRNA (data not shown), or normal cervical specimens (Fig. 1c, f). As summarized in Table 1, there is a strong correlation (74%) between expression of the E5a and c-jun genes.

To analyse the location of E5a and c-jun gene expression, we performed in situ hybridization with an antisense probe. The results show that the signals for E5a mRNA in most HPV-6/11 infected condylomata appear mainly in the basal and parabasal cells (Figs 1a, 2a). Figs 1 and 2 show sagittal and horizontal sections, respectively, of condyloma tissue from a typical specimen. In Fig. 2(a, b), the round areas with lighter stain are dermis tissue. The cells surrounding the dermis tissue are basal cells. It is obvious to us that the darkly stained cells are basal and parabasal cells. The distribution of E5a mRNA is similar to that of c-jun mRNA (Figs 1d, 2b). To exclude the possibility of HPV-6/11 DNA hybridization, specimens were hybridized with the sense E5a or c-jun RNA probe respectively after DNA in the

### Table 1. Correlation analysis between expression of E5a mRNA of HPV-6/11 and c-jun mRNA in genital condylomata

<table>
<thead>
<tr>
<th>c-jun</th>
<th>E5a Positive</th>
<th>E5a Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>42(78%)</td>
<td>4(74%)</td>
<td>46</td>
</tr>
<tr>
<td>Negative</td>
<td>12(22%)</td>
<td>3(5.5%)</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>7(91%)</td>
<td>61</td>
</tr>
</tbody>
</table>

pSL13 produced antisense E5a RNA; T3 promoter driven transcription produced sense E5a RNA. Similarly plasmid JAC.1, which contains a c-jun DNA fragment in pGEM-11, was linearized with NheI or HindIII in the multiple cloning site distal to the T7 or SP6 promoter respectively (Ryder & Nathans, 1988). Transcription driven by the T7 promoter of plasmid JAC.1 produced antisense c-jun RNA; SP6 promoter driven transcription produced sense c-jun RNA. We define probes that have the same polarity as mRNA as sense-strand probes, whereas those in the opposite orientation are antisense-strand probes. In situ hybridization was performed by the method described by Wilson et al. (1992). Radio-labeled riboprobes were synthesized by in vitro transcription. Approximately 300 μCi [35S]UTP was added to each transcription reaction. The specific activities of the probes were about 10^7 c.p.m./μg DNA. The sense-strand RNA probes hybridized to viral DNA but not mRNA, after denaturation of DNA in the specimen by heating in 95% formamide at 65 °C for 15 min. If the DNA in the specimens was not heat-denatured, the antisense-strand RNA probes hybridized to viral mRNA but not DNA. The probe size was reduced to approximately 200 nt in length by controlled alkaline hydrolysis to facilitate penetration into sections and interwending with the target polynucleotides (Cox et al., 1984). In situ hybridization with each of the probes was performed on serial thin-sections of conventional formalin-fixed and paraffin-embedded biopsies of condylomata. High stringency washes, were carried out in 0.1 x SSC (0.15 M NaCl, 0.015 M-sodium citrate) at approximately $T_m -5 ^\circ C$ to eliminate non-specific retention of probes. $T_m$ calculations for RNA:RNA hybrids have been described (Bodkin & Knudson, 1985). According to our previous observations, there is no cross-hybridization among c-jun, junB and junD under highly stringent conditions (Chen et al., 1995). Slides were then dipped in liquefied Kodak NTB-2 radiographic emulsion, exposed at 4 °C for 1 week, and photographically developed in Kodak D19. The sections were counterstained with haematoxylin–eosin, and photographed.

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Fig. 1. E5a and c-jun mRNA expression in condyloma demonstrated by hybridization in situ. Adjacent serial sections of a formalin-fixed, paraffin-embedded condyloma (a, b, d, e) were processed by hybridization in situ using 35S-labelled E5a and c-jun antisense or sense RNA probes as described in the text; (c) and (f) are normal vulvar skin. (a) Treated with E5a anti-sense probe; (b) with E5a sense probe; (c) with E5a antisense probe; (d) with c-jun anti-sense probe; (e) with c-jun sense probe; (f) with c-jun anti-sense probe. Radioactive decay of the probes was visualized as silver grains using bright field illumination. All photographs were taken with a 100 x objective lens.

Fig. 2. Coincidental expression of E5a and c-jun mRNA in a condyloma specimen demonstrated by hybridization in situ. Panels (a, c) and (b, d) are each the same section at a different magnification. (a) and (c) were treated with E5a antisense RNA probe, (b) and (d) with c-jun antisense probe. The photographs of (a) and (b) were taken with a 100 x objective lens, those of (c) and (d) with a 1000 x lens.
specimens was heat denatured (Fig. 1b, e). No hybridization of DNA was detected. There were also no signals for the E5a and c-jun mRNAs in normal vulvar skin (Fig. 1c, f). These observations further established the specificity of the RNA probes. In three out of five E5a mRNA negative condylomata specimens, there were no c-jun mRNA signals (data not shown). However, in two other condyloma specimens without E5a mRNA but which contain c-jun mRNA, the expression of c-jun seems to be independent of E5a induction.

We and other investigators have previously shown that the E5 gene of HPV-11 or HPV-16 can induce expression of c-jun in tissue culture cells (Bouvard et al., 1994; Chen et al., 1994a, b). We also showed by Western analysis that 76.5% of condylomata biopsy specimens exhibit overexpression of the c-jun gene (Yang et al., 1996). Consistently, in this study, we show that the frequency of c-jun gene expression in condylomata is 78% as determined by mRNA hybridization in situ (Table 1). Moreover, the cells expressing c-jun were predominantly in the basal and parabasal cell layers, as were cells expressing E5a (Figs 1 and 2). Similarly, Stoler et al. (1989) reported that a probe for the E5 RNA region of HPV-6/11 detected material in or just above the basal cells in condylomata lesions.

The high frequency of c-jun gene expression in condylomata may be indicative of cell proliferation, since condyloma consists of highly proliferative cells. However, low c-jun gene expression (10.3–39.2%) has been reported in cervical carcinoma, which also consists of highly proliferative cells (Symonds et al., 1992; Yang et al., 1996). These observations suggest that cell proliferation alone may not be able to activate c-jun expression. Interestingly, the low frequency of c-jun gene expression in cervical carcinoma may involve the association between E5 and c-jun gene expression. Since cervical carcinoma containing integrated HPV-16 DNA lacks E5 signals, this part of the viral genome is usually deleted following integration (Pfister & Fuchs, 1994). We believe that this absence of the E5 gene in cervical carcinoma may cause the low frequency of c-jun gene expression.

In this study, we have tried to establish the association between c-jun and E5a in HPV-infected condylomata. This can be best addressed by immunohistochemical analysis using E5a and c-jun antibodies. Since there is no satisfactory E5a antibody available, we used mRNA hybridization in situ to demonstrate expression of E5a and c-jun instead. By using E5a RNA as probe, we cannot rule out concurrent detection of the E1^E4 transcript. The major transcript in HPV-11-infected condylomata cells contains 150 nucleotides from the E7 and E1 ORFs spliced with 1050 nucleotides from the 3' ends of the E2 and E4 ORFs as well as the E5a ORF which potentially encodes the E1^E4 and E5 proteins (Chow et al., 1987). However, it has been reported that E1^E4 protein is localized predominantly in the cell membrane of spinous and granular layers of HPV-11-infected human epithelium (Brown et al., 1994). This location is different from that of the c-jun transcript observed by us in this report. The difference strongly suggests that c-jun is induced by E5a rather than by the product of the E1^E4 transcript of HPV-11.

Our observation of simultaneous expression of E5 and c-jun suggests activation of c-jun by E5a in vivo, an event previously only proven in vitro (Chen et al., 1995). c-jun determines the quantity of AP-1 available for binding to the AP-1 binding site and results in several effects that promote HPV gene expression (Bouvard et al., 1994). One promoting effect may be to further amplify E5 expression, forming a positive-feedback loop; another may activate E6 and E7 expression in the later stages as cells develop from the basal layers to the middle and upper layers (Stoler et al., 1992). Understanding the biological activity of E5 will advance understanding of infection by HPVs and associated neoplasia.

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References


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