Progressive growth of human papillomavirus type 16-transformed keratinocytes is associated with an increased release of soluble tumour necrosis factor (TNF) receptor

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Summary Analysis of conditioned media generated by weakly and highly tumorigenic SKv-1 keratinocyte lines harbouring integrated human papillomavirus type 16 (HPV16) DNA sequences revealed a factor inhibiting TNF-α and TNF-β cytotoxic activity. This inhibitory activity was specifically blocked by htr-9 monoclonal antibody (MAb) recognising 55/60 kDa type I TNF receptor suggesting that it is related to a soluble form of this particular receptor (sTNF-RI). The presence of sTNF-RI was confirmed by Western blot analysis of SKv-1 cell-conditioned medium showing a band of 31.5 kDa as well as by the specific enzyme-linked immunobiological assay (ELISA). Release of sTNF-RI was a result of shedding because Northern blot analysis showed that SKv-1 cells expressed a full-length TNF-RI mRNA, and radioimmunoprecipitation of TNF-RI from [35S]cysteine-labelled cell extracts demonstrated the presence of normal 55 kDa molecule. Evaluation by ELISA showed that highly tumorigenic SKv-12 cells released significantly more sTNF-RI than their weakly tumorigenic SKv-11 parental cells. Furthermore, human recombinant as well as SKv cell-derived sTNF-RI stimulated proliferation of weakly tumorigenic SKv-11 cells. This suggests that a progressive growth of some neoplastic cells may be, at least partially, a result of an increased spontaneous release of sTNF-RI that enables the cells to escape from local TNF-α-mediated growth inhibition.

Keywords tumour necrosis factor; soluble tumour necrosis factor receptor; tumour cell growth; human papillomavirus

Certain types of human papillomaviruses (HPVs) are known to be associated with intraepithelial neoplasia of the cervix and external genitalia (Jenson and Lancaster, 1990; Kiviat and Koutsky, 1993). These lesions usually display a slow, self-limited growth and frequently regress, either spontaneously or after treatment. However, some persisting lesions induced by 'high-risk' HPV types e.g. HPV 16, 18, 31 and 33, may evolve into invasive carcinomas (zur Hausen, 1989; Howley, 1991). The evidence accumulates that growth and dissemination of HPV-associated lesions are under control of local and/or systemic immune surveillance (Jablonska et al., 1989; Pfister, 1990). Especially, local production of immunoregulatory anti-tumour cytokines, e.g. TNF-α, IL-6 and TGF-β, may play an important role. These cytokines were shown to be expressed by HPV-harbouring keratinocytes and may directly or indirectly affect growth of the transformed cells (Woodworth et al., 1990; Majewski et al., 1991; Malejczyk et al., 1991, 1992). Accordingly, a non-tumorigenic SKv keratinocyte cell line established from vulvar intraepithelial neoplasia and harbouring and expressing integrated HPV16 DNA sequences (Schneider-Maunoury et al., 1987; 1990) has been found to release TNF-α spontaneously which in turn exerted an autocrine growth inhibitory effect (Malejczyk et al., 1992). These results strongly suggest the existence of an autocrine TNF-α-mediated mechanism which could be, at least partially, responsible for self-limited growth and eradication of certain HPV-associated tumours.

Progressive growth and dissemination of HPV-induced lesions may be, at least partially, related to escape from local cytokine-mediated surveillance. Indeed, highly tumorigenic HPV-harbouring epithelial lesions found to be resistant to anti-proliferative activity of TGF-β (Woodworth et al., 1990; Braun et al., 1990). Similarly, spontaneous tumorigenic progression of SKv cells was found to be associated with loss of susceptibility to autocrine TNF-α-mediated growth limitation (Malejczyk et al., 1994). This phenomenon was, at least partially, related to a lowered type I TNF receptor (TNF-RI) expression, however, it could also depend on release of some TNF-α inhibitory activity. Therefore, the aim of the present study was to investigate whether increased proliferation and tumorigenicity of SKv cells are associated with an ability to release TNF inhibitory factor(s).

Materials and methods

Reagents Recombinant human TNF-α (rhTNF-α; specific activity 2 x 10^7 U mg^-1) was generously provided by BASF/Knoll (Ludwigshafen, Germany) and recombinant human TNF-β (rhTNF-β; specific activity 3 x 10^7 U mg^-1) was purchased from Genzyme (Boston, MA, USA). Monoclonal htr-9 and utr-1 antibodies specific for type I (55/60 kDa) and type II (75/80 kDa) human TNF-R (TNF-RI and TNF-RII) respectively (Brockhaus et al., 1990), were generously donated by Dr M Brockhaus of F Hoffmann-LaRoche (Basel, Switzerland). Recombinant human type I soluble TNF receptor (rhsTNF-RI; the same as used as a standard for sTNF-RI enzyme-linked immunobiological assay) also originated from F Hoffmann-LaRoche. Radiolabelled [35S]cysteine (specific radioactivity >600 Ci mmol^-1) was obtained from Amersham (Buckinghamshire, UK).
SKv cell lines

SKv keratinocytes harbouring integrated HPV16 DNA sequences were established from vulvar intraepithelial neoplasia. The SKv-1 ('late') cells used in this study display about 10–20 HPV16 genome equivalents resulting from amplifications of a single viral genome insert together with flanking cellular sequences integrated at single chromosome 12q14–q15 (Schneider-Maunoury et al., 1987; Sastre-Garau et al., 1990) and express E6 and E7 viral transforming proteins (Schneider-Maunoury et al., 1990). Two SKv-1 sublines were studied (Maleczky et al., 1994). The first, SKv-11 parental subline, was weakly tumorigenic and after transplantation into nu/nu mice formed the very slowly growing epidermoid cysts with features of Bowen’s carcinoma in situ. The second, SKv-12 line, has been derived from a rapidly growing tumour arising spontaneously after passing of parental SKv-11 cells in nu/nu mice. SKv-12 cells were highly tumorigenic and formed tumours with features of invasive carcinoma. Both SKv-1 cells were propagated in vitro in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 2 mM l-glutamine, 10 mM HEPES and 1% antibiotic–antimycotic solution (GIBCO BRL, Paisley, UK) as described previously (Maleczky et al., 1992).

Evaluation of SKv cell growth

Proliferation of SKv cells either in culture medium alone or with addition of the tested factors was assessed after 48 h by counting the cell number increase in triplicate wells of 24-well tissue culture plates as described (Maleczky et al., 1992, 1994).

Fast protein liquid chromatography (FPLC) fractionation of SKv cell-conditioned media (CM)

For generation of CM, nearly confluent SKv cells were cultured in serum-free culture medium for 48 h. Then cell-free supernatants were harvested, filtered sterilised, and stored frozen at −80°C until used for FPLC fractionation. SKv cell-CM were concentrated about 30–50 times using the Amicon cell with 10 kDa cut-off membrane (Amicon, Danvers, MA, USA). For molecular weight fractionation, 150 μl samples of concentrated SKv cell-CM were subjected to FPLC gel filtration using Bio-Sil TSK guard and Bio-Sil TSK-125 columns (Bio-Rad Laboratories, Richmond, CA, USA). Elution was carried out with phosphate-buffered saline (PBS, pH 7.4) at flow rate 1 ml min⁻¹ and 0.5 ml fractions were collected (Maleczky et al., 1992). For anion-exchange chromatography, 5 ml of concentrated CM was applied onto Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden). Elution was carried out with a continuous 0 to 1 M sodium chloride gradient in 25 mM PIPES buffer (pH 7.2) at flow rate 1 ml min⁻¹ and 0.5 ml fractions were collected. Column fractions were diluted with culture medium and kept frozen until used for further determinations.

Evaluation of TNF inhibitory activity

TNF inhibitory activity in FPLC fractions was evaluated in the presence of 5 U ml⁻¹ of rhTNF-α or rhTNF-β using a conventional TNF bioassay with actinomycin-D-treated L929 cells (Kramer and Carver, 1986). The activity of the inhibitor was expressed in neutralising units (NU), where 1 NU was defined as the highest dilution of the tested material sufficient to neutralise cytotoxic activity exerted by 1 U of TNF-α or TNF-β.

Northern blot analysis

The cells were lysed in 4 M guanidine isothiocyanate containing 0.1 M 2-mercaptoethanol (2-ME) and total RNA was isolated over a caesium chloride cushion. RNA samples were then separated by a gel electrophoresis in 1% agarose under denaturing conditions, transferred onto nitrocellulose membranes and prehybridised as described previously (Maleczky et al., 1992). The presence of TNF-R1 and TNF-RII mRNA was detected by hybridisation, with 32P-labelled probes prepared from 1.0 Kb EcoRI and 0.64 kb NotI–BglII specific cDNA fragments (Smith et al., 1990) respectively (kindly provided by Dr S Gillis, Immunex, Seattle, WA, USA), using RPN 1601 multiprime DNA labelling system (Amersham). After hybridisation, blots were washed in stringent conditions and exposed to X-Omat AR Kodak film at −80°C for 5 days. For control of the RNA amount applied onto each lane, the blots were rehybridised (Maejewski et al., 1991) with 1.15 kb PstI β-actin cDNA probe (Alonso et al., 1983) and exposed for 3 days.

Radioimmunoprecipitation and Western blot analysis

For analysis of cell-associated TNF-R, SKv cells grown in 6 cm plastic dishes (Corning, UK) and were metabolically labelled with 0.5 mCi ml⁻¹ [35S]cysteine in FCS-, methionine- and cysteine-free MEM for 2 h. After labelling, an unincorporated isotope was removed by washing the cells twice in cold TRIS-saline (pH 7.4) and the cells were lysed in 0.5 ml of complete RIPA buffer consisting of 10 mM Tris-HCl, 0.15 M sodium chloride, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 0.2 mM phenylmethylsulphonyl fluoride, and 0.005% aprotinin (pH 7.5) (Schneider-Maunoury et al., 1990). Then the cell extracts were cleared by centrifugation and 200 μl of soluble material was incubated with 10 μg of either htr-9 or utr-1 MAb at 4°C overnight. Precipitation of antigen–MAb complexes was done by incubating with 15 μl of goat anti-mouse IgG covalently linked to Sepharose 4B beads (Zymed, San Francisco, CA, USA) at room temperature for 2 h. After incubation, beads were spun down, washed 4 times in complete RIPA buffer, boiled under reduced conditions in sample buffer consisting of 62 mM Tris-HCl, 10% glycerol, 0.0015% bromophenol blue, 3% SDS and 5% 2-ME (pH 6.8), and subjected to SDS-PAGE as described (Maleczky et al., 1992). The 14C-labelled Rainbow protein molecular weight markers (14.3–200 kDa, Amersham) served as standards. Gels were fixed in 50% ethanol–7% acetic acid, dried and exposed to radioisotope film at −80°C for 7 days.

For detection and characterisation of soluble TNF-R, the SKv cell-CM was extensively dialysed, lyophilised and subjected to SDS-PAGE under reducing conditions as described (Maleczky et al., 1992). Separated proteins were blotted onto nitrocellulose paper (Bio-Rad) and the TNF-R1 and TNF-RII were detected with htr-9 and utr-1 MAb respectively, followed by amplified alkaline phosphatase detection system (Bio-Rad).

Evaluation of sTNF-R

The presence of soluble forms of TNF-R1 and TNF-RII in conditioned media taken from SKv cell cultures after different periods of time was detected and evaluated by specific enzyme-linked immunobiological assay (ELISA) (generously donated by Drs N Drees and H Galati of F Hoffmann-La Roche, Basle, Switzerland). The assays were performed according to the attached manufacturer’s protocols.

Results

Detection of SKv cell-derived TNF inhibitory activity

The presence of TNF-α/TNF-β inhibitory activity in CM generated in 48 h culture of SKv-12 cells was demonstrated by FPLC gel filtration followed by 1929 cytotoxicity assay (Figure 1). Both TNF-α and TNF-β inhibitory activities coeluted as a single peak of 25–35 kDa. This inhibitory
were can Northern blot and containing not the other respectively. As revealed, and utr-1 specifically SKv
fractions from SKv-12 CM were subjected to FPLC gel filtration as described in Materials and methods and serial dilutions of 0.5 ml fractions were tested for TNF inhibitor activity in L929 cell bioassay in the presence of 5 U ml⁻¹ of either rhTNF-α or rhTNF-β (−), OD.

factor was further subjected to anion-exchange FPLC (data not shown), and fractions displaying TNF inhibitory activity were pooled and served as a source of the enriched factor.

To evaluate whether SKv cell-derived TNF inhibitory activity may be related to soluble TNF receptors, fractions containing FPLC-enriched factor were incubated with htr-9 and utr-1 MAb recognising type I and type II TNF-R respectively. As revealed, TNF inhibitory activity was specifically inactivated by htr-9 MAb (data not shown). On the other hand, incubation with utr-1 MAb did not affect the TNF inhibitory activity.

Expression of TNF-R and evaluation of stNF-R release by SKv cells

Expression of TNF-R by SKv-1 cells was studied by Northern blot and radioimmunoprecipitation methods. As can be seen on Figure 2a, hybridisation with TNF-RI cDNA probe showed that both SKv-11 and SKv-12 cells expressed a single 2.5 kb species of specific mRNA. In line with our previous observation (Malejczyk et al., 1994), the levels of specific steady-state mRNA were higher in SKv-11 than in SKv-12 cells. This difference was not caused by an unequal amount of RNA loaded onto each lane in as much as rehybridisation with β-actin cDNA probe showed similar amounts of specific β-actin mRNA. Similarly, radioimmunoprecipitation of TNF-RI from SKv-11 and SKv-12 cell extracts with htr-9 MAb also revealed only a single specific band corresponding to Mₙ of 55 000 (Figure 2b). The amount of TNF-RI was slightly higher in SKv-11 cells.

Neutralisation of SKv-1 cell-derived TNF inhibitory activity by htr-9 MAb strongly suggested that it may be related to stNF-R. Therefore, the presence of stNF-R in SKv cell-CM was evaluated by the Western blot method and specific ELIBA. Indeed, Western blot analysis of SKv-11 and SKv-12 cell-CM showed the presence of a single htr-9-immunoreactive band corresponding to Mₙ of 31 500 (Figure 2c). The intensity of the band was significantly higher in the case of SKv-12 cells. Spontaneous production of stNF-R by SKv-11 cells was further demonstrated by the specific ELIBA (Figure 3). Evaluation of the kinetics of stNF-R release by SKv-1 cells showed that the amount of the factor increased with time of culture reaching a plateau after 48 h. In accord with the results obtained by the Western blot method, there were significant differences (at least at P < 0.01 by Wilcoxon’s test) in stNF-RI release between highly tumorigenic SKv-12 cells and their weakly tumorigenic SKv-11 parental cells. SKv-12 cells released about 2-fold more stNF-R than SKv-11 cells (Figure 3).

Neither the specific TNF-RI mRNA nor TNF-RI protein were detected in SKv-1 cells or SKv-1 cell-CM by Northern blot, radioimmunoprecipitation, Western blot or specific ELIBA (data not shown).

Effect of sTNF-R on proliferation of SKv cells

Compared with weakly tumorigenic SKv-11 parental cells, SKv-12 cells displayed a significantly higher in vitro proliferative potential which correlated with their increased tumorigenic potential (Malejczyk et al., 1994). To evaluate whether the higher proliferation of SKv-1 cells may be related to some stimulatory activity of sTNF-R, both SKv-11 and SKv-12 cells were cultured in the presence of different

Figure 1 Detection of TNF-α (●) and TNF-β (▲) inhibitor in fractions from FPLC gel filtration of SKv-12 CM. Concentrated SKv-12 CM was subjected to FPLC gel filtration as described in Materials and methods and serial dilutions of 0.5 ml fractions were tested for TNF inhibitor activity in L929 cell bioassay in the presence of 5 U ml⁻¹ of either rhTNF-α or rhTNF-β (−), OD.

Figure 2 Detection of (a) TNF-RI mRNA, (b) cell-bound TNF-RI and (c) cell-conditioned medium-derived stNF-RI in SKv-11 (1) and SKv-12 (2) cells. The presence of specific TNF-RI and β-actin mRNA was detected by Northern blot method, cell-bound TNF-RI was detected by radioimmunoprecipitation, and stNF-RI was shown by Western blot, as described in Materials and methods. In both radioimmunoprecipitation and Western blot experiments equal amounts of total protein were applied onto each lane.
concentrations of rhsTNF-RI. As seen in Figure 4, rhsTNF-RI exerted a significant ($P < 0.01$ by non-parametric Wilcoxon test) dose-dependent stimulatory effect on the growth of weakly tumorigenic TNF-sensitive SKv-11 cells. On the other hand, it did not significantly affect the proliferation of highly tumorigenic SKv-12 cells. Heat-inactivated rhsTNF-RI influenced proliferation of neither SKv-11 nor SKv-12 cells.

FPLC-enriched preparation of SKv cell-derived sTNF-RI-related TNF inhibitor stimulated proliferation of SKv-11 cells in a similar way and to a similar extent as rhsTNF-RI (data not shown).

Discussion

The results of the present study show that HPV16-harbouring SKv keratinocytes spontaneously released a factor that protected L929 cells from the cytotoxic activity of both TNF-$\alpha$ and TNF-$\beta$. TNF inhibitory molecules have previously been found in the sera and urine of normal and febrile patients and were identified as soluble forms of TNF-R (Engelmann et al., 1990; Lantz et al., 1990; Seckinger et al., 1990). TNF inhibitory activity of SKv cell-derived factor was specifically neutralised by htr-9 MAb recognising human TNF-RI. This strongly suggests that SKv cell-derived TNF inhibitor is also related to sTNF-RI.

Release of sTNF-RI by SKv-1 cells was confirmed by Western blot analysis and specific ELISA. Upon FPLC gel filtration, SKv cell-derived TNF inhibitor displayed a molecular weight of about 25–35 kDa, and Western blot analysis with TNF-RI-specific htr-9 MAb has revealed a single band of 31.5 kDa. This is in agreement with previous findings on urine-derived sTNF-R as well as sTNF-R released by cells transfected with full-length TNF-R cDNA showing that these molecules display a molecular weight in the range of 27–40 kDa (Engelmann et al., 1989; Kohno et al., 1990; Seckinger et al., 1990). Since only a single major species of specific TNF-R mRNA could be identified in normal and TNF-R cDNA transfected cells (Gray et al., 1990; Kohno et al., 1990; Nophar et al., 1990), it is plausible that these sTNF-R originated from shedding of the membrane-bound receptor. Similarly, Northern blot analysis of mRNA isolated from SKv cells showed a single signal corresponding to full-length TNF-R mRNA. Immunoprecipitation of whole SKv cellular extracts with htr-9 MAb also revealed only one band corresponding to an intact 55 kDa TNF-R. This suggests that sTNF-R released by SKv cells resulted from neither alternative splicing of specific mRNA nor intracellular post-translational modifications of TNF-R1 molecule. Accordingly, SKv cell-derived sTNF-R appears to be an extracellular ligand-binding domain shed directly from the cell surface.

The mechanism responsible for spontaneous shedding of sTNF-R by SKv cells remains unclear. Shedding of sTNF-R was found to be induced by various exogenous stimulatory agents such as phorbol esters, N-formyl Met-Leu-Phe, C5a, GM-CSF and anti-CD3 MAb and requires endogenous protein kinase activity (Gray et al., 1990; Porteu and Nathan, 1990; Crowe et al., 1993). It is plausible that it results from some proteolytic cleavage of cell-bound TNF-R (Nophar et al., 1990), and, in the case of release of sTNF-RII from neutrophils, an elastase is a possible candidate (Porteu et al., 1991).

Highly tumorigenic SKv-12 cells released significantly more sTNF-R than their weakly tumorigenic SKv-11 parental cells. In as much as the amount of the steady-state TNF-R1 mRNA and cell-bound TNF-R1 protein in SKv-12 cells were found to be significantly lower than in SKv-11 cells, this phenomenon cannot be explained simply as being related to a higher level of TNF-R1 expression by SKv-12 cells. The malignant phenotype of a variety of neoplastic cells is associated with increased endogenous protein kinase activity and an excessive release of proteolytic enzymes (Strauli et al., 1980; Bishop, 1991). Accordingly, an increased release rate of sTNF-R1 might be caused by a higher protein phosphorylation rate and/or extracellular protease activity associated with the more malignant phenotype of SKv-12 cells. However, a putative protease that could be responsible for releasing the sTNF-R1 from SKv cells is unknown.

Spontaneous release of sTNF-R/TNF inhibitor has been observed in some tumour cell lines (Aderka et al., 1991). Furthermore, significantly increased levels of sTNF-R were detected in the sera of patients with various neoplastic diseases (Aderka et al., 1991; Waage et al., 1992). Physiological and immunopathological significance of this phenomenon is unknown. However, it is tempting to speculate that sTNF-R may exert a tumour protecting effect: (1) shedding of surface-bound TNF-R from tumour
cells may lead to their desensitisation to circulating TNF, and (2) sTNF-R may neutralise biological anti-tumour activity of the locally released TNF.

Accordingly, release of higher amounts of sTNF-R by highly tumorigenic SK-v-12 cells was accompanied by their higher in vitro proliferation and correlated with their relative TNF-resistance and higher tumorigenic potential (Malejczyk et al., 1994). Furthermore, treatment of weakly tumorigenic, TNF-sensitive SK-v-11 parental cells with rTNF-R1 or sTNF cell-derived TNF inhibitor resulted in a significant dose-dependent stimulation of their growth. This effect was specific and heat-inactivation of rhTNF-R1 resulted in loss of its growth stimulatory effect on SK-v-11 cells. In as much as SKv cells spontaneously express and release TNF-α which, in turn, exerts an autocrine anti-proliferative effect (Malejczyk et al., 1992), the stimulatory effect of sTNF-R1 on SK-v-11 cells may be explained as caused by neutralisation of the endogenous cytokine. Thus, it is plausible that an increased proliferative potential and high tumorigenicity of SK-v-12 cells may be, at least partially, related to an excessive release of sTNF-R1. These results strongly support a view that sTNF-R may enable tumour cell escape from local TNF-α-mediated surveillance and may be one of the important factors facilitating progressive growth of certain neoplastic lesions.

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Abbreviations

CM, conditioned medium; ELISA, enzyme-linked immunobiological assay; FPLC, fast protein liquid chromatography; HPV, human papillomavirus; MAb, monoclonal antibody; NU, neutralising unit; sTNF-R, soluble tumour necrosis factor receptor.

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