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The organotypic culture of HPV-transformed keratinocytes: an effective in vitro model for the development of new immunotherapeutic approaches for mucosal (pre)neoplastic lesions

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Abstract

The purpose of this study is to develop a reliable in vitro human model to test new immunotherapeutic approaches for squamous cell carcinoma that develop on mucosal surfaces. The organotypic (raft) culture permits cells to proliferate and differentiate at an air–liquid interface on a dermal equivalent support. Normal keratinocytes stratify and fully differentiate in a manner similar to the normal squamous epithelial tissues, while human papillomavirus-immortalized and established squamous carcinoma cell lines exhibit dysplastic morphologies similar to (pre)neoplastic lesions seen in vivo. We have demonstrated the ability of these organotypic cultures to be manipulated by altering the epithelial stratification with cytokines (interferon-γ and tumor necrosis factor-α) and by integrating activated lymphocytes or dendritic cells into the in vitro formed epithelial sheet. This model may provide a useful tool to investigate the factors contributing to the presence and function of immunocompetent cells within a neoplastic epithelium that develops on a mucosal surface.

Keywords: Organotypic culture; Human papillomavirus; Immunotherapy

1. Introduction

Cancers associated with potentially oncogenic viruses represent good models for research aimed at developing anti-tumoral vaccines since viral proteins could serve as tumor antigens. One of the most actively investigated examples of a tumor-associated virus is the human papillomavirus (HPV). Infection by specific types of HPV (most notably HPV 16 and HPV 18) is strongly implicated as a causative agent in the etiology of cervical cancer and its precursors, which are designated as cervical intraepithelial neoplasia or squamous intraepithelial lesions (SILs) (for a review, see Ref. [1]). The generation of an effective protective immune response in tumors is, however, still poorly understood and may be influenced by factors produced by neoplastic and infiltrating immune cells. Because different patterns of synergism or antagonism may be observed between these factors, preliminary studies of immunotherapeutic manipulations should be performed in models approximating the in vivo environment of the tissue of origin.

The purpose of this study is to develop a reliable in vitro human model to test new immunotherapeutic approaches for squamous cell carcinoma that develop on mucosal surfaces and particularly in the uterine cervix. The organotypic (raft) culture permits cells to proliferate and differentiate at an air–liquid interface on a dermal-equivalent support. Normal keratinocytes stratify and fully differentiate in a manner similar to the normal squamous epithelial tissues, while HPV-immortalized and established squamous carcinoma cell lines exhibit dysplastic morphologies similar to (pre)neoplastic lesions seen in vivo. The ability of these organotypic cultures to be manipulated may provide a useful tool to investigate the immune factors contributing to eliminate a neoplastic epithelium developed on a mucosal surface.

Interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) are regulatory cytokines with pleiotropic biological activities including antiprolifera-

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tive, antiviral and immunomodulatory activities [2–5]. They are capable of modulating the expression of cell surface molecules, e.g. HLA class II antigens and intercellular adhesion molecules, with a pivotal role in the interactions of neoplastic or infected cells with the host's immune system [4]. The loss of responsiveness to these cytokines by neoplastic keratinocytes might represent a potential mechanism by which the transformed cells escape destruction by the host's immune system.

The most effective mechanism for tumor cell destruction is likely to be cytotoxic T lymphocyte (CTL) responses. T lymphocytes are observed in HPV-associated cervical lesions but there is some evidence that they are poorly activated [6]. Specific CTL against HPV have been detected in T lymphocytes infiltrating cervical lesions, but only in a minority of patients [7]. Professional antigen-presenting cells, such as Langerhans cells, also exhibit quantitative and qualitative alterations in SILs [8–11].

In this study, we demonstrated that the organotypic culture of HPV-transformed keratinocytes is a convenient and bona fide in vitro model to examine the effect of cytokines on the growth of HPV-transformed keratinocytes and the ability of immunocompetent cells to infiltrate a (pre)neoplastic epithelium.

2. Materials and methods

2.1. Culture of normal cervical keratinocytes

Human exocervical epithelial cells were obtained from hysterectomy specimens of healthy women. The operations were performed for disease unrelated to the cervix. Cell cultures were established and maintained following a previously reported method [12]. Subconfluent cultures were dispersed with 0.0025% trypsin and 0.02% ethylenediamine tetraacetic acid. These primary cultures of keratinocytes were used for the organotypic cultures.

2.2. HPV-transformed keratinocyte cell lines

SiHa, CasKi, and C4-II are tumorigenic cervical carcinoma-derived keratinocyte cell lines [13–15]. The SiHa cell line contains one copy of integrated HPV 16 DNA. The CasKi cell line contains approximately 600 copies of integrated HPV 16 DNA, whereas the C4-II cell line contains HPV 18 DNA sequences. The CK2 cell line was established by transfection of human cervical keratinocytes with HPV-33 DNA and is not tumorigenic in nude mice [12]. Two cell lines (supplied by Dr J.K. McDougall) established by transfection of human foreskin keratinocytes with HPV 16 DNA (EIL8; non-tumorigenic) or HPV 18 DNA (18-11S3; tumorigenic after 60 passages in tissue culture) were also tested for response to IFN-γ and/or TNF-α [16]. All these HPV-transformed keratinocyte cell lines were grown and maintained in a mixture of HAM F12 and DMEM supplemented with the same additives as those used for normal keratinocyte cultures.

2.3. Cytokines

Human rIFN-γ (2×10^7 U/mg) and rTNF-α (4.9×10^7 U/mg) were kindly provided by Boehringer Ingelheim International. IFN-γ and TNF-α production in peripheral blood mononuclear cell (PBMC) cultures was determined using an ELISA assay (Medgenix Diagnostics S.A. Fleurus, Belgium).

2.4. PBMC cultures

PBMC were isolated from the buffy coat of healthy donors by centrifugation on Lymphoprep (Nycomed, Oslo, Norway) following standard protocols. For stimulation, cells were cultivated (1.25×10^6 cells/ml) in the presence of 10 ng/ml anti-CD3 mAb kindly provided by Dr Kurrle Behringwerke (Marburg, Germany) and/or 50 U/ml human rIL-2 kindly provided by Glaxo Institute for Molecular Biology (AG, Genève, Switzerland) during 4 days.

2.5. Dendritic cell cultures

Dendritic cells (DC) were generated by culturing adherent fractions of human PBMC as previously described [17]. The phenotype of the cells was verified by flow cytometric cell surface marker analysis using anti-CD14, anti-CD1a, anti-CD4 and anti-CD3 antibodies.

2.6. Organotypic cultures

Organotypic cultures of normal cervical keratinocytes and HPV-transformed keratinocytes were prepared by using procedures slightly modified from those described previously [16]. For the preparation of dermal equivalents, a collagen matrix solution was made with 32 mg collagen (cellagen solution AC-5, type I; ICN Biomedical, Asse-Relegen, Belgium) mixed on ice with 1.6 ml of 0.1% acetic acid, 1 ml chilled tenfold concentrated Hank's buffer supplemented with phenol red and 1 N NaOH to give pH 7.2. One milliliter of fetal calf serum containing 4×10^5 normal human fibroblasts was then added. One milliliter of the collagen-fibroblasts solution was poured into 24-well plates (Nunclon Δ Multidishes, NUNC, Denmark) and allowed to solidify at 37°C for 1H. The final concentrations of collagen and fibroblasts were 3.2 mg/ml and 4×10^4 cells/ml, respectively. After gel equilibration with 1 ml growth

medium overnight at 37°C, 25×10^4–1×10^6 keratinocytes (normal or HPV-transformed) resuspended in 100 μl growth medium were seeded on top of the gels and maintained submerged for 24–96 h. The collagen rafts were raised in a 25 mm tissue culture insert (8 μm pore size; NUNC, Denmark) and placed onto stainless-steel grids, at the interface between air and liquid culture medium. Epithelial cells were then allowed to stratify for 10–15 days. Hematoxylin eosin staining and standard immunohistological techniques with antibodies against markers of cell proliferation (MIB-1; Immunotech S.A.), differentiation (involucrin; Novocastra), cell adhesion (E-cadherin, HECD-1; Boehringer Ingelheim International) were first used to assess the degree of similarity between the in vitro formed epithelial sheet and the in vivo observed epithelium. We next determined whether these organotypic cultures can be manipulated. For the cytokine experiments, medium added or not with either IFN-γ (1000 U/ml), TNF-α (1000 U/ml) or IFN-γ (1000 U/ml) in combination with TNF-α (1000 U/ml) was changed every 2–3 days. Organotypic cultures then were fixed in 10% neutral buffered formaldehyde, paraffin-embedded, sectioned and stained with hematoxylin and eosin or immunolabeled with the MIB-1 monoclonal antibody specific for Ki-67 antigen. For other experiments, the PBMC and DC were seeded on top of the in vitro formed epithelium after stratification of keratinocyte. The PBMC and DC concentrations were 10^6 cells/50 μl and 2×10^5 cells/50 μl growth medium, respectively. For the DC experiments, organotypic culture medium was supplemented or not with 800 U/ml granulocyte macrophage-colony stimulating factor (GM-CSF). After 24–72 h at 37°C, the collagen rafts were harvested. The cultures were then embedded in O.C.T. compound (Tissue Tek, Sakura, Netherlands) at −70°C and sectioned with a cryostat microtome for the immunohistochemical analysis. The density of T lymphocytes and DC migrating into the epithelial layer was assessed by a standard avidin–biotin–peroxidase technique with monoclonal antibodies specific for CD3, CD45 (Becton Dickinson, Erembodegem, Belgium) and CD1a (clone NA1/34 from Dako, Glostrup, Denmark).

2.7. MTT assay

HPV^+ cell lines were seeded at a concentration of 2.5×10^3 cells/microwell in 200 μl culture medium containing 100 or 25 μl PBMC culture supernatant. The cells were incubated 4 days at 37°C and 5% CO_2. In each well, 10 μl cell proliferation reagent WTS-1 (tetrazolium salt; Boehringer Mannheim) were added and incubated for 4 h at 37°C and 5% CO_2. The absorbance of the sample was measured at 440 nm with a microtiter plate reader.

3. Results

3.1. Assessment of the degree of similarity between the in vitro formed epithelial sheet and the in vivo observed epithelium

After 10–15 days, normal keratinocytes produced differentiated epithelial layers of about ten cells in thickness morphologically similar to a normal squamous exocervical epithelium (Fig. 1A,B), whereas HPV-transformed keratinocytes produced an epithelial sheet of up to 10–15 cells in thickness [18] (Fig. 1C). These cells appeared disorganized and highly atypical throughout its full thickness, reminiscent of high-grade cervical lesions (Fig. 1D). Ki-67 antigen was expressed throughout the full thickness of the epithelium, as already observed in biopsy specimens of high-grade SIL [9] (Fig. 1E,F). In contrast, the normal cervix and normal keratinocyte raft cultures showed important staining for the differentiation marker, involucrin, whereas this staining was absent or limited to the superficial cell layers for both HPV^+ keratinocyte raft cultures and (pre)neoplastic lesions of the cervix (data not shown). There was also a decreased cell membrane staining of the cell adhesion molecule, E-cadherin, in SILs of the cervix and in organotypic cultures of HPV-transformed keratinocytes compared with the normal exocervical epithelium and organotypic cultures of normal keratinocytes (data not shown).

3.2. Effect of IFN-γ and TNF-α on normal and HPV-transformed keratinocytes in organotypic cultures

The growth of normal keratinocytes in organotypic cultures was inhibited by IFN-γ, TNF-α and IFN-γ associated to TNF-α. This antiproliferative effect of cytokines was reflected by the impairment of normal epithelial stratification that was reduced to a few cells in thickness. The stratification and immunostaining for Ki-67 antigen of HPV-transformed keratinocytes were dramatically decreased in the presence of IFN-γ and IFN-γ associated to TNF-α (Fig. 2A,B). A more pronounced effect was observed with the combination of IFN-γ and TNF-α. TNF-α alone, at a concentration of 1000 U/ml, did not significantly alter the stratification-proliferation of the HPV-transformed cell lines [18].

3.3. Infiltration of PBMC into organotypic cultures of HPV-transformed keratinocytes

After 24 h of co-culture with the keratinocyte rafts, mononuclear cells (detected by anti-CD45 mAb) were observed in the epithelial sheet. The majority of these cells were T lymphocytes as shown by the anti-CD3 labeling (data not shown). The density of IL-2 + anti-CD3 mAb-stimulated PBMC infiltrating the epithelial

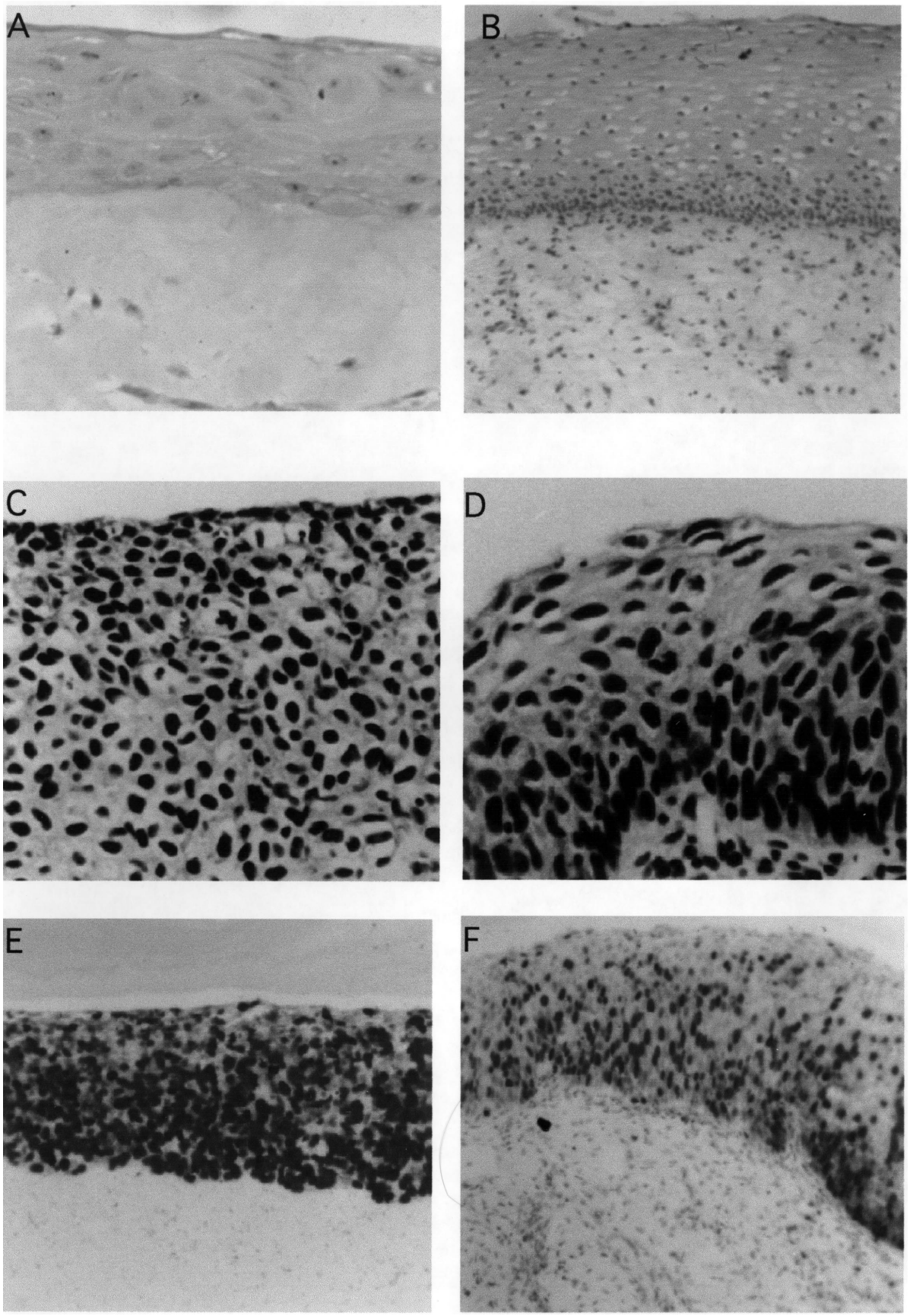

Fig. 1. Normal and HPV-transformed keratinocyte organotypic cultures as models of normal squamous epithelium and high-grade cervical lesions, respectively (A–D: hematoxylin–eosin). (A) Section of an organotypic culture of normal cervical keratinocytes. (B) Biopsy specimen of normal exocervical squamous epithelium. (C) Section of an organotypic culture of SiHa cells. (D) Biopsy specimen of high-grade cervical SIL. (E–F) Ki67 antigen-immunostained sections of an organotypic culture of HPV-transformed keratinocytes (E) and a biopsy specimen of high-grade cervical lesion (F).

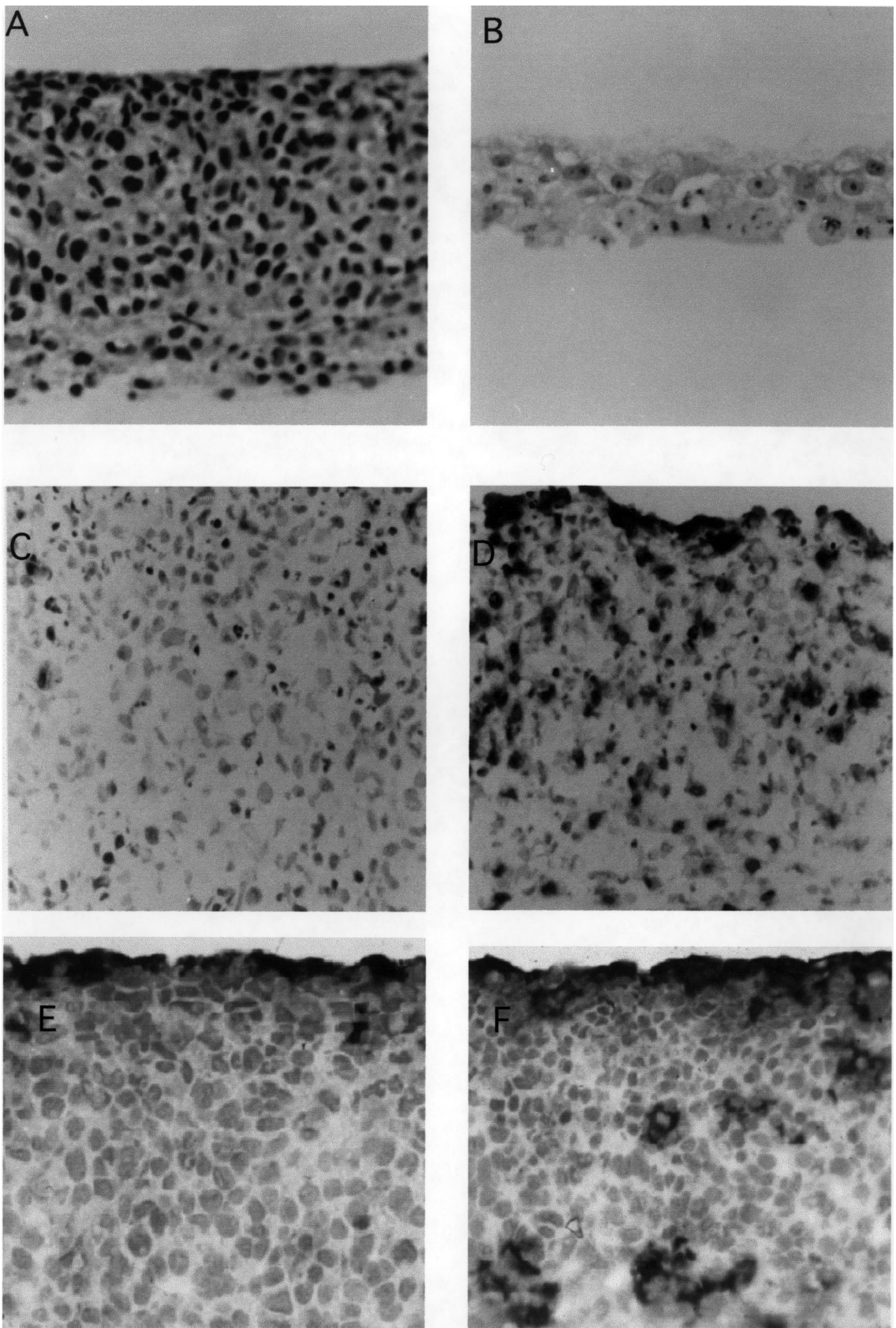

Fig. 2. Representative examples of organotypic cultures of HPV-transformed keratinocytes supplemented by cytokines and/or infiltrated by immunocompetent cells. Hematoxylin–eosin stained sections of organotypic cultures of SiHa cells in the absence (A) or in the presence (B) of IFNg associated with TNFa. CD45-immunolabeled sections of organotypic cultures of SiHa cells infiltrated by resting PBMC (C) or by PBMC stimulated with IL-2 + anti-CD3 (D). CD1a-immunolabeled sections of organotypic cultures of SiHa cells infiltrated by dendritic cells in the absence (E) or in the presence (F) of GM-CSF.

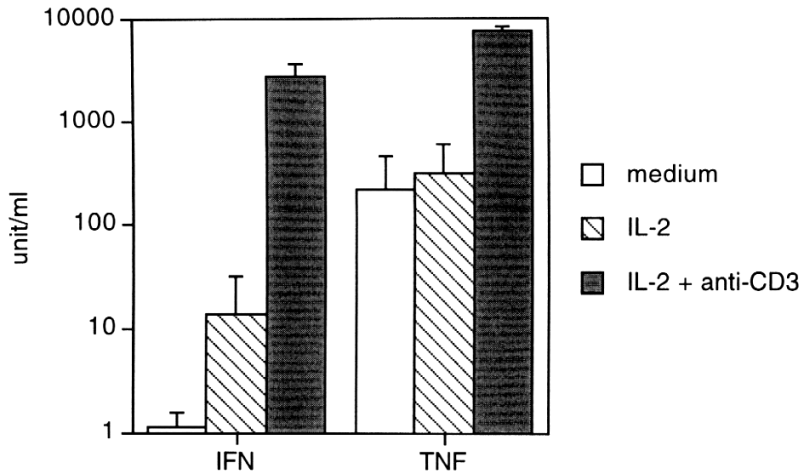

Fig. 3. IFN-γ and TNF-α production in cultures of unstimulated (medium) or stimulated PBMC (IL-2 or IL-2 + anti-CD3 mAb).

sheet was higher than that of unstimulated or IL-2-stimulated T lymphocytes (Fig. 2C,D). These PBMC had a cytotoxic effect since apoptotic keratinocytes were detected in organotypic cultures [19]. Interestingly, IL-2 + anti-CD3 mAb-activated PBMC produced high amounts of IFN-γ and TNF-α (Fig. 3), and the supernatant of these cultures inhibited the proliferation of HPV^+ cell lines (Fig. 4), suggesting that IFN-γ and TNF-α could participate in the cytotoxic effect observed in organotypic cultures.

3.4. Infiltration of DC into organotypic cultures of HPV-transformed keratinocytes

Fig. 2E–F illustrates representative experiments showing the density of CD1a-labeled DC in HPV-transformed organotypic cultures incubated with or without exogenous GM-CSF. In organotypic cultures of normal keratinocytes, DC migrated in a similar manner in the presence or in the absence of GM-CSF [20]. In contrast, DC layered onto organotypic cultures of SiHa poorly infiltrated the epithelial layer in the absence of GM-CSF (Fig. 2E), whereas addition of GM-CSF caused an important increase in the density of DC observed in the epithelial layer (Fig. 2F).

4. Discussion

Because the validation of new treatments based on the stimulation of the immune system of the host may benefit from studies performed with in vitro models mimicking many in vivo features, we developed an organotypic culture system that permits epithelial cells to proliferate and differentiate at an air–liquid interface on a dermal equivalent support. Normal keratinocytes stratify and fully differentiate in a manner similar to the normal squamous epithelial tissues, while HPV-immortalized and established squamous carcinoma cell lines exhibit dysplastic morphologies similar to (pre)neoplastic squamous lesions seen in vivo.

Morphological and immunohistological techniques with antibodies against markers of cell proliferation, differentiation, and cell adhesion were first used to assess the degree of similarity between the in vitro formed epithelial sheet and the in vivo observed epithelium. Our results demonstrated that the organotypic (raft) culture is not simply constituted by a random accumulation of cells, but also reproduces faithfully normal or (pre)neoplastic epithelial tissues, as observed in vivo. We next demonstrated the ability of these

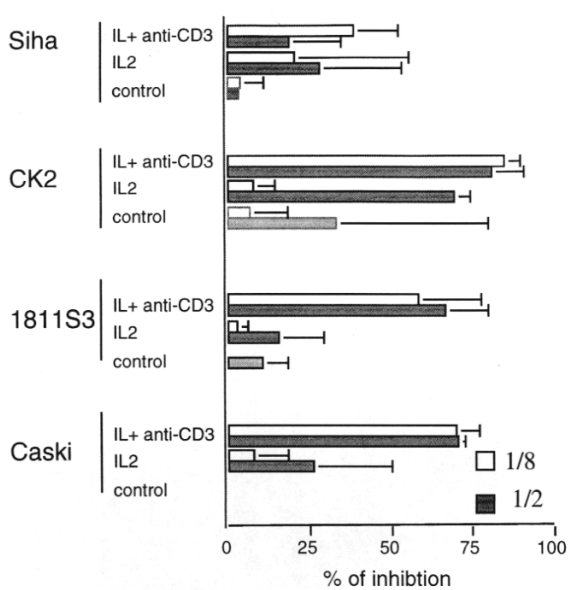

Fig. 4. Inhibition of HPV^+ cell line proliferation by culture supernatants of unstimulated (medium) or stimulated PBMC (IL-2 or IL-2 + anti-CD3 mAb). Two dilutions of supernatants were used (1/2 and 1/8). Means ± S,D, of two or three independent experiments are shown.

organotypic cultures to be manipulated. We showed that HPV-transformed keratinocytes do not lose their sensitivity to the antiproliferative effect of IFN-γ alone or associated with TNF-α, compared with normal keratinocytes. These results associated to the demonstration of a localized immunodepletion in HPV-related lesions [10,21] suggest that these recombinant cytokines might be useful in the treatment of cervical HPV-associated cervical lesions. We also succeeded to integrate in these in vitro formed epithelial sheets some types of immunocompetent cells. Since intra-epithelial T lymphocytes have been shown to be depleted in cervical SIL [22,23] and to increase in spontaneously regressing HPV-associated genital warts [24], the cell-mediated immune response probably plays a crucial role in host defence against HPV infection and associated-(pre)cancerous lesions, perhaps via the production of IFN-γ and TNF-α. The establishment of an in vitro model allowing for the investigation of the factors contributing to the presence and function of immunocompetent cells within a dysplastic epithelium is therefore relevant. Since tissue cells cultivated on plastic are in an environment that does not prevail in vivo, organotypic cultures present a very promising in vitro model for testing the ability of immune cells to infiltrate a pre-neoplastic epithelium. To evaluate the feasibility of this model, as a first step, we have studied the infiltration of resting and activated allogeneic PBMC into an organotypic culture of HPV-transformed keratinocytes. The PBMC were activated by IL-2 and IL-2 + anti-CD3 to allow the generation of LAK cells, as already used in immunotherapeutical protocols [25]. The activation in the presence of anti-CD3 mAb induced an increase in the proportion of T lymphocytes as we previously showed [26]. Although unstimulated and stimulated lymphocytes were able to penetrate SiHa organotypic cultures, the stimulated lymphocytes infiltrated the cultures more rapidly and in higher numbers, especially after activation with IL-2 + anti-CD3 mAb. Most of the infiltrating lymphocytes were T cells. These results highlight the potential interest of organotypic cultures of HPV-transformed keratinocytes as a model for studying the ability of lymphocytes to migrate into a (pre)neoplastic epithelium. Other models are, however, necessary to analyse the ability of lymphoid cells to cross the basement membrane, such as those using a three-dimensional collagen matrix [27,28].

As most pre-neoplastic lesions of the cervix are associated with a significant depletion of intra-epithelial Langerhans cells, when compared with normal cervix epithelium [8,9,11], we also investigated the migration of in vitro generated DC in the presence of keratinocytes derived from normal cervix and HPV-transformed cell lines in organotypic cultures. We and other workers have observed that HPV^+ keratinocytes cell lines produce less GM-CSF than normal keratinocytes [29]. Since GM-CSF is an essential factor not only for the maturation and differentiation of DC but also for their motility [30], we tested the capacity of this cytokine to influence the ability of DC to colonize an in vitro formed (pre)neoplastic epithelium. Under basal conditions, the level of DC infiltration in cultures of normal keratinocytes was higher than that of HPV^+ cell lines cultures. Except for one cell line, a positive correlation was observed for the numbers of $CD1a^+$ cells present in the epithelial layer and the amount of GM-CSF produced by the HPV^+ cell lines (data not shown). When the medium of organotypic cultures of HPV^+ cell lines was supplemented with GM-CSF, the infiltration of DC improved and reached an infiltration level equivalent to that obtained with normal keratinocytes. These findings suggest that a treatment based on GM-CSF may restore some immunologic functions that have been shown to be altered during the progression of cervical SILs.

In conclusion, this study highlights the potential interest of the organotypic culture of HPV-transformed keratinocytes for the design of new immunotherapeutic strategies. The ability of this model to be manipulated (for example, by integrating cytokines and/or immunocompetent cells) may also provide a useful tool to investigate the factors contributing to the presence and function of immunocompetent cells within a neoplastic epithelium.

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