Virology 385 (2009) 192–197

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Expression of the human papillomavirus type 16 E7 oncoprotein induces an autophagy-related process and sensitizes normal human keratinocytes to cell death in response to growth factor deprivation

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ARTICLE INFO

Article history:
Received 3 September 2008
Returned to author for revision
30 September 2008
Accepted 2 December 2008
Available online 8 January 2009

Keywords:
Human papillomavirus
Keratinocytes
Autophagy
Cell death
E7 oncoprotein
Trophic sentinel signaling
Metabolic stress

ABSTRACT

Expression of oncogenes, such as the human papillomavirus type 16 (HPV16) E7 oncoprotein, promotes aberrant cell proliferation. In the absence of concurrent mitogenic stimuli, this triggers a cell-intrinsic defense mechanism, the "trophic sentinel response", which eliminates such aberrant cells. The molecular pathways that elicit this response, however, remain obscure. We set up an experimental system to investigate the trophic sentinel pathway triggered by HPV16 E7 expression in normal human keratinocytes, the natural host cells of HPVs. Keratinocytes expressing HPV16 E7 cultured in E-medium undergo cell death and show increased sub-G1 DNA content when grown to confluence or under conditions of serum deprivation. Moreover, HPV16 E7 expressing human keratinocytes express higher levels of the autophagy marker, LC3-II, which can be abrogated by 3-methyladenine, an autophagy inhibitor. These findings indicate that even under normal culture conditions, HPV16 E7 expression triggers metabolic stress that may result in autophagy, a pathway implicated in carcinogenesis.

Introduction

Normal cells undergo growth arrest when they reach confluence and/or are deprived of growth factors. Cells that have suffered a single oncogenic hit, however, can encounter a situation of conflicting growth signals when the oncogenic proliferative signal is inconsistent with environmental antiproliferative cues. As a consequence, such cells undergo an abortive process, the "trophic sentinel response", which can result in cell death, differentiation or senescence (reviewed in Evan and Vousden, 2001). This phenomenon was initially discovered in cells expressing the adenovirus (Ad) E1A oncogene (Debbas and White, 1993; Putzer et al., 2000; Rao et al., 1992; Teodoro et al., 1995; White et al., 1991), or c-*myc* (Evan et al., 1992). Co-expression of Ad E1B or Bcl-2 in Ad E1A or c-*myc* expressing cells, respectively, mutes this response and results in cellular transformation (Debbas and White, 1993; Pelengaris et al., 2002; White et al., 1991). Hence, the trophic sentinel response is thought to represent a cellular tumor suppressive process that eliminates aberrantly proliferating cells from an organism (reviewed in Evan and Vousden, 2001).

High-risk human papillomaviruses (HPVs) are etiological agents of cervical carcinoma and are also associated with other anogenital tract malignancies as well as head and neck cancers (reviewed in Schiffman et al., 2007). Due to frequent viral genome integration during malignant progression, high-risk HPV associated cancers consistently express the two viral oncoproteins, E6 and E7. The best known cellular targets of high-risk HPV E6 and E7 oncoproteins are the p53 and retinoblastoma (pRB) tumor suppressor proteins, respectively (reviewed in Munger et al., 2001).

Our group showed that IMR90 normal human diploid fibroblasts that express the high-risk HPV type 16 (HPV16) E7 oncoprotein are predisposed to undergo cell death once the cells become confluent and/or are serum starved (Jones et al., 1997b). Induction of the trophic sentinel response by HPV16 E7 correlated with its ability to destabilize the retinoblastoma tumor suppressor pRB and to stabilize the p53 tumor suppressor. This effect was abrogated when the HPV16 E6 oncoprotein, which targets p53 for degradation (Scheffner et al., 1993; Scheffner et al., 1990), or a dominant negative p53 mutant was co-expressed (Eichten et al., 2004). Although caspase 3 is activated and DNA fragmentation occurs in serum starved HPV16 E7 expressing IMR90 cells, treatment with a pan-caspase inhibitor did not block cell death in HPV16 E7 expressing IMR-90 cells. Hence, the trophic sentinel response in HPV16 E7 expressing normal human fibroblasts depends on p53, but does not involve prototypical apoptosis (Eichten et al., 2004) and requires additional mechanistic investigation.

To analyze the trophic sentinel response in a biologically relevant cell type, normal human keratinocytes, we developed an assay system where normal human keratinocytes are grown in E medium supplied with 5% fetal bovine serum (FBS). We show that under these

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doi:10.1016/j.virol.2008.12.003

conditions, normal human epithelial cells undergo growth arrest upon serum deprivation or when the cells reach confluence. In contrast, HPV16 E7 expressing keratinocytes undergo cell death as evidenced by an increase in the sub-G1 population. Furthermore, we detected evidence of autophagy in HPV16 E7 expressing keratinocytes, even when the cells were grown under normal tissue culture conditions. These findings will allow a detailed mechanistic investigation of the trophic sentinel response triggered by HPV16 E7 expression in keratinocytes and suggest that E7 expression results in metabolic stress even under normal tissue culture conditions.

Results

HPV16 E7 expressing primary human foreskin keratinocytes are prone to cell death upon serum deprivation in E medium

Our previously published experiments on the HPV16 E7 induced trophic sentinel pathway were performed with normal human fibroblasts rather than epithelial cells, the normal host cell type for HPVs. In order to investigate the mechanistic details of the trophic sentinel pathway in normal human keratinocytes, we first needed to develop a workable experimental system. Normal human keratinocytes are most conveniently cultured in serum free growth media such as Keratinocyte Serum Free Medium (K-SFM) (Pirisi et al., 1987), which is supplemented with pituitary extract and recombinant EGF. Upon depletion of the pituitary extract from K-SFM, normal human keratinocytes failed to undergo a cell cycle phase specific growth arrest (J. Hasskarl and K. Münger, unpublished) whereas EGF depletion inhibited cell migration but increased their proliferative capacity (Hasskarl et al., 2006). Based on these results, we explored alternative growth conditions for human keratinocytes. E medium, based on Dulbecco's modified Eagle medium (DMEM) supplemented with F-12 nutrient mixture and a variety of other components such as insulin, transferrin, adenine, triiodothyronine and hydrocortisone, as well as fetal bovine serum (FBS) supports the growth of normal human keratinocytes (Rheinwald, 1980; Rheinwald and Beckett, 1980). While the original formulation also requires the presence of mitomycin C treated Swiss 3T3 J2 murine fibroblasts or their conditioned medium, we did not include these components for our studies.

Primary human foreskin keratinocytes (HFKs) cultured in E medium showed decreased cell growth in response to serum deprivation as evidenced by lower cell density after 48 h of treatment (data not shown). Cell cycle analysis by FACS revealed a modest increase in the G1/S ratio, from 1.9 to 2.7, representing cell growth arrest, but no apoptotic cells with sub-G1 DNA content were detected at 72 or 144 h after serum withdrawal (Fig. 1A, left panels). In order to determine whether these culture conditions may be used to analyze trophic sentinel signaling, we subjected a passage and donor matched population of HPV16 E7 expressing HFKs to the same treatment. There was no marked decrease in cell density after 48 h of serum deprivation in E-medium as compared to cells grown in E-medium supplemented with 5% fetal bovine serum (FBS) (data not shown). FACS analysis revealed an increase of cells in S and G2/M phases with a concomitant decrease in G1 phase cells. Most importantly, there was a dramatic increase of cells with sub-G1 DNA content from 0.2% at 72 h to 10% at 144 h after serum deprivation (Fig. 1A, right panels). Similar results were obtained in three independent experiments. Hence, HPV16 E7 predisposes human foreskin keratinocytes to cell death in response to growth factor withdrawal, similar to what we previously observed with normal human diploid fibroblasts (Jones et al., 1997b).

HPV16 E7 expressing hTERT immortalized human oral keratinocytes are predisposed to cell death upon serum deprivation

Given that HFKs have a limited lifespan and their growth characteristics change upon repeated passaging, they need to be re-

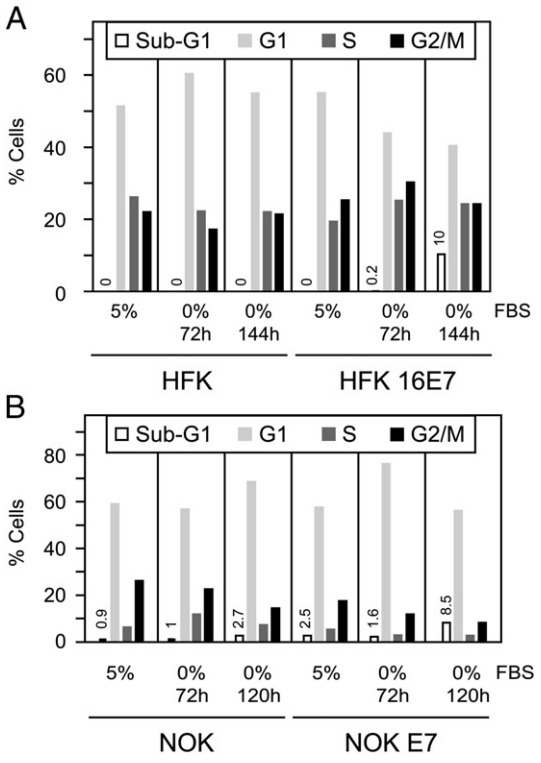

Fig. 1. HPV16 E7 expressing normal human keratinocytes cultured in E medium are prone to cell death upon serum deprivation. (A) Representative flow cytometric cell cycle analysis of control human foreskin keratinocytes (HFK) and HPV16 E7 expressing populations (HFK 16E7) grown in E-medium with 5% FBS or in serum free E medium for 72 and 144 h. Similar results were obtained in three independent experiments. (B). Representative flow cytometric cell cycle analysis of telomerase immortalized normal human oral keratinocytes (NOK) and a donor matched HPV16 E7 expressing line (NOK E7) (Piboonniyom et al., 2003) grown in complete E-medium or in serum free medium E medium for 72 and 120 h. Similar results were obtained in three independent experiments.

derived regularly. Due to genetic differences between individual donors, there can be significant differences between cell populations. To minimize these effects and to further confirm that the trophic sentinel pathway is functional in normal human keratinocytes, we investigated a set of matched hTERT immortalized normal oral human keratinocyte lines (NOK) with and without HPV16 E7 expression (Piboonniyom et al., 2003). As with the primary HFKs, these cells were adapted to grow to E-medium containing 5% FBS. After serum withdrawal for 24 and 48 h, we observed increased cell shrinkage and rounding in NOK E7 cells (data not shown). To quantify this phenotype, we performed FACS analysis after serum withdrawal for 72 h and 120 h (Fig. 1B). In NOK cells we observed an ~10% increase in the G1 population, with concomitant decreases in the S and G2/M populations. There was only a minor increase in the population of cells with a sub-G1 DNA content from 0.9% to 2.7%. In NOK E7 cells, however, we observed an increase in the sub-G1 population from 1.6% to 8.5% at 120 h after serum deprivation (Fig. 1B). Similar results were obtained in three independent experiments.

Increased incidence of cell death in HPV16 E7 expressing human keratinocytes upon cell–cell contact

In the course of these experiments, we observed that the overall incidence of cell death upon serum deprivation of NOK E7 cells was dependent on the saturation density that the cells reached. We consistently observed a higher number of floating cells in dense cultures of NOK E7 cells than in NOK control cells, even when the cells were maintained in E medium supplied with 5% FBS. Hence, we investigated whether E7 expression can trigger trophic sentinel