DYNAMICS OF DIFFERENTIATION IN HUMAN EPIDERMOID SQUAMOUS CARCINOMA CELLS (A431) WITH CONTINUOUS, LONG-TERM θ-IFN TREATMENT

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SUMMARY

We investigated the long-term effects of continuous gamma interferon (θ-IFN) treatment on A431, a human squamous carcinoma cell line. Cells were grown in an in vitro culture system, which over time produces cohesive cell masses ("tumoroids") exhibiting three-dimensional, histotypically differentiated structures, e.g., keratin "pearls," intercellular bridges (desmosomes), elongated flattened cells (squares) and stratification. The effects of θ-IFN on cell growth, morphology and stage of differentiation were assessed at different treatment times by light and electron microscopy and by immunohistochemical staining using antibodies to keratins 1 and 14 and to filaggrin, markers of specific stages of keratinocyte differentiation. Our results show that A431 cells have the capacity for spontaneous differentiation, that this capacity is significantly enhanced and accelerated by θ-IFN treatment leading to terminal differentiation and extensive cell death by 2 wk. Despite continuous exposure to IFN, a small number of viable, undifferentiated cells remain. Their proliferation, evident by 3 wk, reconstitutes the tumoroid which once again contains the full range of differentiating cell types.

Key words: human squamous carcinoma cells (A431); θ-IFN; differentiation; long-term in vitro culture.

INTRODUCTION

A431 is a human epidermoid squamous carcinoma cell line (14). In an in vitro three-dimensional culture system (16,27,28) cohesive cell masses ("tumoroids") develop which exhibit organizational features typical of stratified squamous epithelium. These include numerous, prominent desmosomes ("intercellular bridges"), keratin "pearls," and elongated flattened cells (squares) which often assume a parallel orientation (stratification).

The synthesis of keratin intermediate filaments is highly regulated in stratified squamous epithelia, with the appearance of characteristic keratins in specific layers during development and differentiation. In vivo, K-14 is synthesized primarily in the actively proliferating cells of the basal cell layer; K-1 is found only in suprabasal layers in cells committed to differentiation (9,13,23,32); and filaggrin, a protein indicative of cornification, is synthesized in the granular cell layer (10,11).

Interferons (IFNs) have been shown to have antiviral, antiproliferative, immunomodulatory, cytotoxic, and differentiating effects in in vitro systems (1,4,7,12,15,17,19,21,24,26,30,31). θ-IFN treatment of monolayer cultures of A431 cells results in a marked inhibition of proliferation, morphologic changes, and extensive cell death (5). Terminal differentiation rather than cytotoxicity was suggested as the cause of cell death when immunohistochemical staining of 5-day tumoroids showed that, compared to untreated controls, IFN-treated cultures contained greater numbers of cells with both the phenotype and high molecular weight keratins associated with fully differentiated cells (6).

In the present study we examined the effects of continuous θ-IFN exposure on monolayer and long-term tumoroid cultures using the keratin markers of differentiation. We found that the temporal sequence of keratin gene expression in tumoroids follows the same orderly progression as in vivo and was not changed by IFN treatment. We observed that the cell death seen with IFN treatment in

![Graph](image_url)
monolayer culture also occurs in tumoroid cultures and is due to a substantial increase in the number of differentiating cells and an acceleration of their rate of terminal differentiation. Not all treated cells, however, enter the differentiation pathway. A small number of stemlike cells continue to divide despite continuous exposure to γ-IFN, and their proliferation results in a repopulated tumoroid that, once again, contain cells in various stages of differentiation.

**MATERIALS AND METHODS**

**Cell cultures.** A431 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in 150-cm² flasks in Dulbecco’s modified Eagle’s medium (DMEM) (Bio-Fluids, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) (BioFluids), 1.0% glutamine, and 1.0% pen-strep (of 5000 U/ml penicillin and 5000 µg/ml streptomycin). At 70 to 80% confluence, cells were washed in phosphate buffered saline (PBS), treated with trypsin (0.25%-EDTA (1 mM EDTA) in Hanks’ balanced salt solution (NIH media unit), counted, and resuspended in media for tumoroid and monolayer cultures.

**Agar discs.** One gram of Bacto-agar (Difco Laboratories, Detroit, MI) was added to 100 ml Earle’s balanced salt solution (NIH media unit) with 100 µg/ml gentamicin (GIBCO, Grand Island, NY), autoclaved at 250 °C for 30 min and slightly cooled. Two-mill