Intranuclear Localization of Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3) During Cell Division in Human Keratinocytes

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Insulin-like growth factor-I (IGF-I) stimulation of basal keratinocytes is an essential component of normal epidermal homeostasis. In addition to the IGF receptor, basal keratinocytes synthesize insulin-like growth factor binding protein-3 (IGFBP-3). The HaCaT keratinocyte cell line, which has many characteristics of basal keratinocytes, synthesizes IGFBP-3 that in vitro reduces its IGF-I responsiveness. IGFBP-3 has attracted interest as a potential growth arrest protein, both via its ability to modulate IGF-I responsiveness, and more controversially via IGF-I-independent mechanisms. Intracellular modes of action have been proposed, and a nuclear localization consensus sequence has previously been identified within IGFBP-3. Using immunocytochemistry with a biotinylated antibody specific for IGFBP-3, we investigated the intranuclear localization of IGFBP-3 in subconfluent monolayer cultures of HaCaT cells. Diffuse cellular staining was visible, potentially corresponding to cell surface and nascent cytoplasmic IGFBP-3. Of particular interest however, was the localization of staining over the nuclei of a large proportion of cells that were undergoing cell division. Antibody staining was specific for IGFBP-3 because addition of recombinant human IGFBP-3 to the antibody prior to incubation with the cells inhibited these staining patterns. Optical sections obtained using a confocal laser scanning microscope showed that in keratinocytes undergoing cell division, IGFBP-3 was localized inside the nucleus. These results show that intracellular IGFBP-3 localization is altered during the cell cycle and suggest a possible nuclear role for IGFBP-3 during cell division. Key words: cytokine/IGF-I/mitosis/nucleus. J Invest Dermatol 111:239–242, 1998

MATERIALS AND METHODS

Reagents
An affinity-purified, biotinylated goat anti-serum specific for human IGFBP-3, and which specifically recognizes the 38–42 kDa IGFBP-3 protein in western analysis (Berchet et al, 1995; Conover et al, 1995; Hasegawa et al, 1995), was generously provided by Dr. C.J. Strasburger (Ludwig-Maximilians-Universität, Munich, Germany). An affinity-purified rabbit anti-Ki67 antibody was purchased from DAKO (Glostrup, Denmark). Trypsin, Dulbecco’s modified Eagle’s medium, and fetal bovine serum were purchased from Trace Biosciences (Clayton, Australia).

Cells
The immortalized human keratinocyte cell line, HaCaT (Boukamp et al, 1988), was kindly provided by Prof. N. Fusenig (German Cancer Research Center, Heidelberg, Germany). Cells at passage numbers 43–45 were maintained as monolayer cultures in 5% CO2 at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. At confluence, cells were detached by incubating with 4 mM ethylenediamine tetraacetic acid followed by 0.025% trypsin, 0.5 mM ethylenediamine tetraacetic acid, then seeded onto autoclaved glass microscope slides. Cells were grown to 90% confluence, washed in phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Fixed monolayers were permeabilized and dehydrated through graded ethanol and then stored desiccated at 4°C.

Immunostaining
Fixed cells on glass slides were boiled in a microwave oven in 10 mM sodium citrate, pH 6.0, for 10 min then washed three times in PBS at room temperature. All subsequent steps were performed at room temperature. Slides were incubated in methanol containing 1% hydrogen peroxide, then in PBS containing 1% bovine serum albumen, 20 µg sheep IgG (Silus, Hawthorn, Australia) per ml for 30 min each. Slides were then incubated in PBS containing 0.1% bovine serum albumen and the biotinylated anti-human IGFBP-3 (13 µg per ml) for 3 h. To test for immunospecificity, the antibody was incubated with 15 µg recombinant human IGFBP-3 (Upstate Biotechnology, NY) per ml at room temperature for 30 min before applying...
Keratinocyte nuclei stain positive for IGFBP-3 by immunostaining. A subconfluent monolayer culture of HaCaT human keratinocytes was fixed in paraformaldehyde, permeabilized, and immunostained with an affinity-purified, biotinylated antibody specific for human IGFBP-3, counter-stained in hematoxylin as described in Materials and Methods, and then viewed under light-field microscopy. (a) IGFBP-3-positive nuclei are indicated by the brown immunostaining. The nuclei of cells undergoing division are clearly positive for IGFBP-3 staining (arrows). In nondividing cells, cellular (non-nuclear) staining is visible. Scale bar, 40 µm. (b) Immunostaining is blocked by the inclusion of 15 µg recombinant human IGFBP-3 per ml with the antibody incubation, showing that the staining patterns were specifically due to the presence of IGFBP-3. Scale bar, 50 µm. (c) Incubation with a biotinylated goat IgG antibody as a negative control for the primary antibody showing the specificity of the detection system for the presence of IGFBP-3. Scale bar, 50 µm.

Immunofluorescence Fixed cells were treated and incubated at 4°C overnight with the biotinylated anti-IGFBP-3 antibody as described above, then after washing the slides in PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (12 µg per ml) followed by anti-streptavidin (5 µg per ml) and finally FITC-streptavidin were from Vector Laboratories. All incubations were for 30 min at room temperature, washing with PBS between each incubation. Slides were mounted in Mowiol (Hoechst, Frankfurt, Germany) containing 2.5% 1,4-diazobicyclo[2.2.2]octane (DABCO) antifade (Sigma) and viewed with a Leitz Dualplan fluorescence microscope.

Confocal microscopy Cells were immunostained as described above and viewed on an Optiscan F900e fiber optic laser scanning confocal microscope (Optiscan, Melbourne, Australia) with a 60× lens (numerical aperture = 1.4) and a 1300 nm diameter optical fiber (equivalent to a pin-hole size of 1300 nm), producing a z-axis resolution of 1 µm.

RESULTS

Keratinocyte nuclei stain positive for IGFBP-3 during cell division Analysis of HaCaT keratinocyte monolayers using an antibody specific for human IGFBP-3 showed that most cells had diffuse cytoplasmic and/or surface IGFBP-3, indicated by the brown staining (Fig 1a). Of particular interest, however, was the detection of a small but significant proportion of dividing cells with intense brown staining in the region of the cell nucleus (Fig 1a). This staining pattern was specific for IGFBP-3, as shown by the lack of both nuclear and non-nuclear staining when antibody binding is blocked by the addition of recombinant IGFBP-3 (Fig 1b). Figure 1(c) is a negative control showing no staining when a biotinylated preimmune goat IgG is used instead of the IGFBP-3-specific antibody.

The localization of IGFBP-3 in the keratinocytes was also analyzed using immunofluorescence to determine if a fluorescein-conjugated antibody detection system produced the same staining pattern as that shown in Fig 1. As shown in Fig 2(a), nuclear fluorescence was clearly visible in dividing cells. Other nondividing cells were visible that exhibited diffuse extranuclear fluorescence only (Fig 2a). Figure 2(b)–(d) are negative control monolayers that also contained several dividing keratinocytes per field, showing abolition of IGFBP-3 immunostaining with (i) the coaddition of recombinant IGFBP-3 (Fig 2b), (ii) incubation with biotinylated preimmune goat IgG instead of specific antibody (Fig 2c), and (iii) an absence of antibodies or FITC-conjugated streptavidin (to exclude the possibility of autofluorescence) (Fig 2d).

IGFBP-3 is inside the nucleus during keratinocyte division To ensure that the apparent nuclear staining with the IGFBP-3 antibody was not due to IGFBP-3 present in perinuclear structures such as, for example, Golgi apparatus or secretory vesicles, laser scanning confocal microscopy was performed on keratinocyte monolayers that had been prepared as described above for immunofluorescence. This technique has the ability to differentiate between proteins located within or peripheral to the nucleus by taking thin optical sections through subcellular structures. Optical sections (1 µm thick) through the nuclei of dividing keratinocytes consistently demonstrated the presence of IGFBP-3 throughout the nucleus (Fig 3).

Monolayers of HaCaT keratinocytes contain a high proportion of dividing cells To determine the proportion of actively dividing keratinocytes present in the HaCaT monolayer cultures, the monolayers were immunostained with an antibody specific for the cell cycle associated nuclear antigen, Ki67. Ki67 is expressed most abundantly during M phase but is also detectable in G1, S, and G2. Counting of several representative fields showed that monolayers contained ~10% Ki67-positive cells (Fig 4). Monolayers matching those in Figs 1–3 thus contain a significant proportion of actively dividing cells.
paraformaldehyde, permeabilized, and incubated with a polyclonal rabbit antibody specific for Ki67. (a) Actively dividing cells in the monolayer are indicated by the presence of brown immunostained nuclei. Scale bar, 50 µm. (b) A negative control (rabbit preimmune serum) for the Ki67 antibody shows no staining. Scale bar, 50 µm.

Figure 4. Sub-confluent keratinocyte monolayers contain a high proportion of dividing cells as detected by Ki67 immunostaining. Sub-confluent monolayers of HaCaT human keratinocytes were fixed in paraformaldehyde, permeabilized, and incubated with a polyclonal rabbit antibody specific for Ki67. After incubation with FITC-conjugated streptavidin, cells were analyzed by scanning laser confocal microscopy. The figure shows IGFBP-3 immunofluorescence in a 1 µm thick optical section through dividing keratinocytes. Scale bar, 10 µm.

DISCUSSION

IGFBP-3 has been studied extensively as an IGF-I carrier in serum and as a modulator of IGF-I action at the cell surface of target tissues (Jones and Clemmons, 1995). A potential role for IGFBP-3 in cell growth arrest has been supported by recent data that the IGFBP-3 promoter contains an active response element for the tumor suppressor gene p53 (Buckbinder et al, 1995), that expression of the IGFBP-3 gene caused premature growth arrest in transfected mouse fibroblasts (Cohen et al, 1993), and that IGFBP-3 appears to mediate the growth inhibitory effects of transforming growth factor-β and retinoic acid in a human breast cancer cell line (Oh et al, 1995; Gucev et al, 1996).

Other studies have identified growth inhibitory mechanisms of IGFBP-3 (Valenti et al, 1995; Rajah et al, 1997) and of proteolytic fragments derived from IGFBP-3 (Lalou et al, 1996) that appear to be independent of IGF receptor signaling. Data consistent with the existence of a cell surface receptor for IGFBP-3 (Oh et al, 1993) also suggest that IGFBP-3 growth inhibitory mechanisms may go beyond a role in inhibiting the interaction of IGF-I and its receptor at the cell surface.

The identification of a nuclear localization consensus sequence in IGFBP-3 (Radulescu, 1994) originally highlighted the possibility that IGFBP-3 has an intracellular function, potentially in gene regulation. Our demonstration of intranuclear IGFBP-3 in keratinocytes suggests that these as yet unknown functions may be important in the regulation of epidermal growth. If nuclear IGFBP-3 has a biologic function, the question of whether nuclear IGFBP-3 works via an IGF–dependent or IGF–independent mechanism remains. During the preparation of this manuscript, two reports on nuclear IGFBP-3 in other cell types were published, one of which reported the transport of transport of IGF-1/IGFBP-3 complexes to the nucleus of opossum kidney cells (Li et al, 1997). Consistent with our own data, nuclear transport only occurred in actively dividing cells. In the other report, nuclear IGFBP-3 was detected in a lung cancer cell line (Jaques et al, 1997). A feature of this study is that the thin optical sectioning technique of confocal microscopy effectively distinguishes between intranuclear IGFBP-3 and IGFBP-3 that may be merely associated with the nucleus, nuclear envelope, or adherent perinuclear organelles.

There are two possibilities for the cellular origin of nuclear IGFBP-3 in keratinocytes: (i) a certain proportion of nascent IGFBP-3 is diverted from its secretory pathway to a nuclear transport pathway at cell division, or (ii) extracellular IGFBP-3 is internalized and transported to the nucleus at cell division. It remains to be seen at which stage of the cell cycle this translocation occurs. Fibroblast growth factor-1, another secreted protein with a nuclear localization signal (Imamura et al, 1990), is known to translocate to the nucleus during late G1 phase of the cell cycle (Imamura et al, 1994). It is interesting to observe that the immunostaining pattern of nuclear IGFBP-3 differs slightly from that of Ki67. This could suggest either that Ki67 and IGFBP-3 appear in the nucleus at different stages of the cell cycle, or that they have different intranuclear distributions. Further studies are required to pinpoint the exact phase at which nuclear IGFBP-3 appears in keratinocytes.

Further studies will also be required to investigate the occurrence of nuclear IGFBP-3 in the epidermis in vivo. Whereas our inspections of skin sections have not so far shown nuclear IGFBP-3 localization in the basal cells of the human epidermis, which is the in vivo localization of IGFBP-3 in the epidermis (Batch et al, 1994; Wright et al, 1997), this is perhaps not surprising, considering the small proportion of basal keratinocytes undergoing cell division in vivo. Ki67 staining, which identifies any cells not in G0 phase, stains only about one in 50 cells of the basal epidermis in our studies (data not shown). This correlates with the published observations of Hodak et al (1996). Of these cells, which are in any of G1, S, G2, or M phases, only a small proportion will be in M, and then only a small proportion of these will be undergoing cytokinesis, the stage at which IGFBP-3 appears to localize in the nucleus of keratinocytes.

The paracrine interaction of IGF-I from the dermis (Minuto et al, 1991; Tavakkol et al, 1992) with the IGF receptor in basal keratinocytes (Hodak et al, 1996) is known to be essential for the normal development of the differentiated epidermal layers (Liu et al, 1993). Our previous studies have shown that IGFBP-3 expression in the basal layer is likely to play a key role in this interaction (Wright et al, 1994, 1997). This study indicates that IGFBP-3 in basal keratinocytes could have far more complex mechanisms of action than previously envisaged in the control of epidermal growth.

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REFERENCES


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