Effects of Insulin-like Growth Factor Binding Protein 7 on Apoptosis in Human Teratocarcinoma Cells In Vitro

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Abstract. Human teratocarcinoma cells (Tera-2) deprived of serum undergo programmed cell death which can be counteracted by simultaneous addition of IGF-II. This protective effect of IGF-II was specific in the sense that addition of IGF-binding protein 7 (IGFBP-7) resulted in an increased apoptotic rate almost comparable to that of the classical IGFBPs. Autoradiographic analysis of incorporated tritiated thymidine indicated that the proportion of S-phase cells was comparable, irrespective of total cell numbers. This further suggests that IGF-II rescues cells from apoptosis and that IGFBP-7 is a specific antagonist.

Human teratocarcinoma cells, and in particular the cell line Tera-2 (1), grown in vitro are dependent on exogenous insulin like growth factor I or II (IGF-I and IGF-II) for exponential growth (2, 3). It was revealed that the IGFs do not act in a cell cycle-specific manner, i.e. by controlling entry into the cell cycle from quiescence. Instead, it has been shown that the IGFs specifically support cell survival. Thus, Tera 2 cells supported by IGFs have been shown to be a useful system for studying apoptotic mechanisms. When IGF-binding protein 2 (IGFBP-2) was added to Tera-2 cell grown in serum free medium, the antiapoptotic effect was abrogated in a concentration dependent fashion (4). In addition to the classical insulin-like binding proteins 1-6 there exists a class of IGFBP-related proteins, IGFBPPrPs, (5) which share high homology with IGFBPs but have a lower affinity for IGFs than that of IGFBP-1-6 (6). One of these is generally known as IGFBP-7 (7). This study has aimed at examining the effects of this archetype IGFBPPrP on cell multiplication and apoptosis in Tera-2 cells. We report here that in spite of their differences in IGF-binding capacity, the canonical IGFBP-2 and IGFBP-7 have similar effects on counteracting the antiapoptotic effects of IGF II.

Materials and Methods

Cell culture. Tera 2 cells were maintained and flasks and wells for experimental purpose were essentially prepared as previously described (12). All tissue culture material was obtained from NUNC (Roskilde, Denmark). The serum-free medium consisted of a 1/1 (v/v) mixture of α−modified Minimal Essential Medium (MEM) lacking nucleosides and deoxynucleosides and Hams F12 medium supplemented with 10 μg/ml of human transferrin preloaded with iron, in accordance with the manufacturers instructions. This medium is subsequently referred to as α:Ham.

Two days before a growth experiment, the medium over the stock cultures was replaced by α:Ham supplemented with 10% heat inactivated foetal calf serum (FCS). This procedure allowed the cells to adapt to the basal medium that was used in the serum-free culture experiments. The cells were removed from the stock culture flasks by rinsing with phosphate-buffered saline (PBS) and then briefly exposing the cells to a mixture of trypsin, versene and chick plasma (TVP). The cells were taken up in a:Ham with 10% FCS and counted in a Coulter counter (Coulter Electronics, Bedford, UK). In the subsequent cell culture experiments, the media were pre-equilibrated for at least six hours in an atmosphere of 5% (v/v) CO2 in 95% humidified air at 37˚C. The day before the experiment, the cells were plated out in 10 ml of α:Ham with 10% FCS at a density that ranged between 1.4×10⁵ and 3.0×10⁵ cells per 60-mm diameter Primaria dish (Becton Dickinson, San Jose, Ca, USA). The number of cells plated out varied between experiments, but numbers were always comparable within each experimental series. At the start of each experiment, cells were rinsed twice in PBS and 10 ml of α:Ham were added to each dish. After one hour, the dishes were briefly removed from the incubator and the IGFs and binding protein added to the dishes. Two dishes were counted immediately after the PBS rinse to obtain a starting cell count, and on subsequent days, the effect of the additives was measured by counting duplicate dishes in each treatment. For these counts, the cells were exposed to trypsin (0.125%, w/v) and EDTA in PBS for up to 30 minutes to ensure complete detachment from the cell surface. When all cells

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had detached, any remaining trypsin was neutralised by adding soybean trypsin inhibitor. The cells were then counted in triplicate in a Coulter counter. The range of the cells of the duplicate results rarely exceeded 8% of the mean cell count.

**DNA synthesis and assessment of apoptosis.** DNA synthesis was assayed by incorporating tritiated thymidine (Amersham, Sweden) to 25 Ci/mmol into cells growing on glass coverslips for one hour prior to fixation. Unincorporated thymidine was removed after fixation for 24 hours in 50/50 methanol/acetic acid (v/v) by washing with ice-cold trichloroacetic acid. The cells were then processed for autoradiography as described elsewhere (8). The assessment of intact vs apoptotic cells was performed by acridine orange staining and fluorescence microscopy as described elsewhere (9). Nuclear fragmentation corresponded to nucleosome laddering and FACS analysis as previously described in detail (9).

**Growth factor and binding proteins.** Recombinant IGF-II was purchased from British Biotechnology (Oxford, UK). Lyophilised samples were made up to stocks of 2 mg/ml by adding 0.5 ml of 0.1 M acetic acid to 10 mg, shaking every 5 minutes in a 37°C water-bath and then adding 4 ml of PBS with 1% (wt/vol) crystalline bovine serum albumin (BSA; Sigma, Stockholm, Sweden) adjusting the pH to 7.0 with 0.1 M NaOH, and finally bringing the volume up to 5 ml with PBS/BSA. These stocks were aliquoted into Eppendorf vials and stored at –70°C until further use. In the growth experiments, the IGF-containing vials were thawed on ice, and made up to intermediate stock solutions by diluting with α:Ham to a final concentration of 200 μg IGF-II/ml medium. 0.5 ml of this intermediate stock solution was added to each 60 mm dish containing 10 ml serum free α:Ham. Dishes used for experimental purposes were harvested after 24 hours. In each case, serum-free α:Ham and α:Ham supplemented with 10% serum were used as controls.

IGFBP-7 was purchased from R & D systems (MN, USA) and stock solutions made up to the relevant concentration (50 ng/ml) according to the manufacturer’s instructions.

**Table I. Effect of IGF-binding protein 7 on cell number.** Cells were grown for up to 6 days in a:Ham medium with supplements (10% FCS, 30 ng IGF-II/ml or 50 ng IGF-binding protein 7/ml (BP7)). To ensure a continuous supply, the appropriate amount was added daily. Cells were counted in triplicate in a Coulter counter each day as indicated. Each value is from two different experiments and is the mean±SD (n=6).

<table>
<thead>
<tr>
<th>Day</th>
<th>10% FCS</th>
<th>10% FCS +BP7</th>
<th>IGF-II</th>
<th>IGF-II+BP7</th>
</tr>
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<tbody>
<tr>
<td>1</td>
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<td>161±25</td>
<td>161±25</td>
<td>161±25</td>
</tr>
<tr>
<td>4</td>
<td>388±108</td>
<td>350±76</td>
<td>288±40</td>
<td>179±43</td>
</tr>
<tr>
<td>5</td>
<td>514±98</td>
<td>391±100</td>
<td>405±65</td>
<td>155±32</td>
</tr>
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<td>6</td>
<td>643±102</td>
<td>455±78</td>
<td>511±112</td>
<td>186±43</td>
</tr>
</tbody>
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It is generally acknowledged that IGF II promotes Tera-2 cell multiplication by counteracting programmed cell death induced by exposure to serum-free medium. Addition of IGFBP-7 together with IGF II, reversed the survival effect of IGF-II and instead increased the proportion of fragmented nuclei (Table II). These data were further corroborated by data on the proportion of S-phase cells in each experimental situation, irrespective of cell numbers as presented in Table III. An 1 hour pulse treatment with tritiated thymidine yielded percentages of labelled cells in the interval 25-33%, i.e. figures that did not in any case differ significantly from one another.

**Discussion**

The size of a cell population is controlled by the relative rates of proliferation, differentiation and cell death. It is nowadays an established fact that programmed cell death - apoptosis - plays a pivotal role in control of normal cell proliferation. Apoptosis normally occurs during embryonic development, particularly when complex organs are formed, where there is much cell turnover and selection during ontogeny. For example, neurones that have failed to reach their target cell type die in the CNS. Likewise, self-reactive T-cells are eliminated via the thymus. Apoptosis is a common phenomenon in tumours, even more so in rapidly growing tumours. This suggests massive cell turnover, but another plausible explanation is that genes that control the onset of apoptosis may have been selected for.

The IGFs play a pivotal role in the control of normal embryogenic development. But an equally important role has been attributed to these growth factors in tumourigenesis (10-12). IGF-II appears to play several roles in these processes. Firstly it supports the maintenance of proliferation via the type 1 IGF receptor which acts as a proper signal-mediating
transducer. But secondly, IGF-I and -II play a spurious role in the control of apoptosis. This is particularly striking in the case of Tera-2 cells, where the normal control mechanisms for exit from or entry into the cell cycle have been lost. But it is also clear that the IGFs can exert the opposite effect and initiate apoptosis in Wilms tumour cells (10).

The concentration of IGF in the vicinity of the cell is controlled by specific binding proteins as well as by a specialised scavenger receptor (type 2 IGF receptor) that act in concert to ensure a finely tuned supply of growth factor to the cells. The discovery of modified IGFBPs with a decreased IGF binding capacity (e.g. IGFBP6Ps) of which IGFBP-7 is the archetype, further adds to the complexity of this picture (7). IGFBP-7 has been shown to be a potential tumour suppressor for prostate cancer cells (13). This binding protein can even delay the G1-phase in tumour cells and force tumour cells into apoptosis via the cyclin A pathway (14). Moreover, it is a clear antagonist to VEGF induced angiogenesis (15). IGFBP-7 also exerts different roles in the regulation of melanoma (16) and glioblastoma (17, 18) cell proliferation. Eventhough the IGFBPs can exert the opposite effect and initiate apoptosis in Wilms tumour cells. Mol Pathol 48: M153-M157, 1995. However, at least this proportion of labelled cells was scored and data expressed as a percentage. At least 300 cells were counted per coverslip. Each value is the mean of two different experiments.

**Acknowledgements**

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**References**


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**Table II. Effect of IGF BP-7 on apoptotic figures.** Cells were grown in media with IGF-II and/or IGFBP-7 as described in Materials and Methods. Daily a coverslip from each experiental setup was stained with acridine orange and viewed in a fluorescence microscope. The proportion of apoptotic versus intact nuclei was scored and data expressed as the percentage of intact cells. At least 300 cells were counted per coverslip. Each value is the mean of two different experiments.

<table>
<thead>
<tr>
<th>Day</th>
<th>SFM</th>
<th>10% FCS</th>
<th>10%FCS +BP7</th>
<th>IGF-II</th>
<th>IGF-II+BP7</th>
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<td>32</td>
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</tbody>
</table>

SFM: Serum-free medium.

**Table III. Effect of IGFBP-7 (BP7) on the proportion of S-phase cells.** Cells were grown in media with IGF-II and/or IGFBP-7 as described in Materials and Methods. Daily a coverslip from each experiental setup was pulse labelled with tritiated thymidine and thereafter subjected to autoradiography. The proportion of labelled cells was scored and data expressed as a percentage. At least 300 cells were counted per coverslip. Each value is the mean of two different experiments.

<table>
<thead>
<tr>
<th>Day</th>
<th>SFM</th>
<th>10% FCS</th>
<th>10%FCS +BP7</th>
<th>IGF</th>
<th>IGF+BP7</th>
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<tbody>
<tr>
<td>1</td>
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SFM: Serum-free medium.


