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ORIGINAL ARTICLE

Expression and Growth Dependency of the Insulin–like Growth Factor I Receptor in Basal Cell Carcinoma

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Basal cell carcinoma is the most common skin cancer, accounting for ~70% of all skin malignancies. The actual risk of metastasis for these tumors is exceedingly rare, but they are locally aggressive, and 5–9% may have multiple recurrences. Generally, growth hormone promotes local release of insulin-like growth factor I (IGF-I), which in turn activates the IGF-I receptor (IGF-IR). In this study we tried to find out whether IGF-IR may be involved in basal cell carcinoma growth. To address this issue, we analyzed mouse basal cell carcinoma ASZ001 cells and normal keratinocytes HaCaT cells for IGF-IR expression and IGF-I dependency using alamar blue which is reduced to a fluorescent pink dye by the metabolic activity of living cells. We found in IGF-1 treated cells, a increase alamar blue reduction compared with control cells suggesting a certain degree of stimulatory response in these cells. Therefore, IGF-1R is a key molecule with roles common to both the carcinogenesis and normal tissue development pathways. Our data points to the possibility of the involvement of IGF-IR inhibitors as a treatment modality. Further studies on larger collections of cases are needed to evaluate the expression level and distribution of IGF-IR and its correlation to different histopahological subtypes.

Keywords: basal cell carcinoma, IGF-IR, alamar blue

Introduction

In present, basal cell carcinoma is the most common cutaneous malignant tumor. The highest rates are in Australia, followed by USA, where every year are estimated over 1 million new cases diagnosed from the total of cancer diagnosed cases of other types of combined cancer [1,2,3]. The incidence rates of a number of countries or European regions are substantially lower – 150 / 100 000 per year in Europe [4]. Although basal cell carcinomas typically occur in adults, the tumours also develop in children [5].

Data from special literature state that lower rates of metastases are of 0.028% in dermatological institutions and 0.1 in surgery departments [6,7]. BCC is aggressive firstly due to local destruction determined by tumor's invasion on profound states. Knowing that this tumor has a preferential localization for cephalic extermination, many times, the surgical excision is incomplete, for which the tumor has the possibility to invade more into surrounding tissues, destroying or canceling the patient's vital functions like hearing, seeing and smelling, for instance [8].

In countries with a high incidence of basal cell carcinomas it is not unusual to have individuals with multiple basal cell carcinomas, and regular review is required to deal with new skin tumours. Incomplete removal of basal cell carcinoma may result in delayed recurrences that may not be recognized for years, particularly if the tumour recurrence is deep or masked by skin grafts [9].

It is well known that the main stimulatory effects of growth hormone on the growth of normal epithelial cells are exerted through the IGF-I system [10,11,12,13]. In the skin, IGF-1 is produced by cells of mesenchymal origin, such as fibroblasts of the dermis and dermal papilla, whereas its receptor is produced by both mesenchymal and epithelial cells [14,15,16,17]. Thus, keratinocytes respond to the paracrine signal originating from the neighboring mesenchymal cells.

Insulin-like growth factor 1 (IGF-1) is a peptide hormone that promotes growth, survival, and differentiation of cells in various organs and tissues, including skin [18,19]. Although generally recognized as a proliferation and survival factor for the skin, IGF-1 was recently also implicated in hair and skin morphogenesis [20,21]. In the skin, its levels must be strictly controlled because overexpression of IGF-1 in proliferating and in differentiating keratinocytes resulted in hyperplasia and tumor formation [22,23]. In vitro, IGF-1 was shown to stimulate keratinocyte proliferation and migration, as well as collagen production by fibroblasts [24,25,26,27].

Epidemiological prospective studies identified high plasma levels of IGF–1 as potential risk factor for several malignancies [28,29]. In addition, the IGFs are a potent mitogen for a wide range of tumor cell types in vitro [30,31,32]. IGF–1R is involved not only in the induction of cell transformation but also in the maintenance of the transformed phenotype [33].

Aim

The specific aim of our study is to evaluate the expression status and possible dependency of IGF-I receptor (IGF-IR) in promoting the growth of basal cell carcinoma and to describe the characteristics of alamar blue method in order to define the optimal culture conditions for mouse basal cell carcinoma cell line ASZ001.

Materials and methods

Materials

Dulbeco's modified Eagle's medium (DMEM) and heat inactivated fetal bovine serum were obtained from Gibco (Life Technologies, Belgium). Penicillin–streptomycin solution, gentamicin solution, trypsin solution were from Invitrogen (LifeTechnologies, Merelbeke, Belgium). Alamar Blue was purchased from Biosource (Biosource Euroupe, Nivelle Belgium), IGF1 from Sigma-Aldrich (Bornem, Belgium).

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Cell Culture Protocol

The mouse basal cell carcinoma cell line ASZ001 [34,35] and HaCaT [36] cells were maintained in 75 cm2 Falcon culture flasks under standard culture conditions of 5%CO2 in air, 95% relative humidity and 37°C with medium renewal every 2–3 days. The culture media used was DMEM containing 200 mM glutamine, 1/100 penicillin–streptomycin solution and 1/1000 gentamicin solution supplemented with 10% fetal bovine serum. When confluent, cells were split 1 : 3.

Cells were trypsinized from subconfluent cultures by adding 3 ml of trypsin solution (100 mg EDTA, 100 mg DNase I, 5 ml penicillin–streptomycin, 500 µl gentamicin in 500 ml trypsin) to 50 ml Falcon flasks with confluent cells followed by 10 min incubation at 37°C with regular gentle shaking. The trypsin reaction was stopped by adding 10 ml of appropriate culture medium containing 10% fetal bovine serum. The cell suspension was then centrifuged at 750 g for 10 min at 20°C. The cell pellet was suspended in 2 ml of medium with 1% fetal bovine serum and thoroughly mixed by repeated pipetting. Cells were then counted in a Countess Automated Cell Counter (Invitrogen LifeTechnologies, Merelbeke, Belgium) using trypan blue staining according to the manufacturer's protocol.

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Alamar Blue assay for cell quantification

The suspension were seeded into triplicate wells of a 96-well plate (200 µl well-1) at concentrations of 1 x 10^4–4x10^4 cells/well at standard culture conditions of 5% CO_2 in air at 37°C. After an initial 4 h period to allow cell attachment, 20 µl alamar blue solution was directly added to the medium resulting in a final concentration of 10%. As negative control alamar blue was added to the medium without cells. The plate was further incubated for 24 h at 37°C. The absorbance of test and control wells was read at 540 and 630 nm with Infinite M1000 (Tecan Spectra, Germany)- a new class of multimode microplate reader at 24, 48 and 72h after adding alamar blue.

The number of viable cells correlates with the magnitude of dye reduction [37,38,39]. The values of alamar blue reduction were corrected for background values of negative controls containing medium without cells. Data are given as means of triplicate measurements performed in three independent experiments.

Alamar Blue assay for cell viability/proliferation

Next, the viability of HaCaT and mouse basal cell carcinoma cell line ASZ001 cells was tested in the presence of IGF-I. Cells were seeded into 96-well plates at a concentration of 1x104 cells/well for 24 h in a standard incubator. The cells were allowed to attach and grow for 24 hours. They were then serum-starved for 12 hours and then they were supplement with serum or stimulated with IGFI (50 ng/mL) in the absence of serum for 12 hours.

Alamar Blue was added directly into culture media at a final concentration of 10% and optical densities were measured after yet another 4 h period. The absorbance was read at 567 and 585 nm with Infinite M1000 (Tecan Spectra, Germany) using Tecan's universal data analysis - Magellan software.

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Results

Growth characteristics of basal cell carcinoma in culture HaCaT is the first permanent epithelial cell line from adult human skin that exhibits normal differentiation and provides a promising tool for studying regulation of keratinization in human cells. As no human BCC cell lines are available, we extensively tried to establish human BCC cell lines. However, we could neither obtain transplantable BCC nor establish BCC cell lines. So we used a mouse BCC cell line, ASZ001.

Alamar Blue reduction showed high reproducibility with very low intra-assay and inter-assay cell viability. There was also a good linear correlation between alamar blue reduction and cell concentrations over a range of 1x104cells/well - 4x104cells/well both at 24h and 48h both for HaCaT (data are not shown) and for ASZ001 cells (Figure1). The initial experiments were carried out with coloured cultured media. Interference from medium colour was excluded by negative controls containing medium with serum without cells.

Furthermore, it has been shown that proteins in culture media can cause spectral shifts and may affect the kinetics of the assay e.g. the rate of uptake of substrate by the cells. Therefore, caution is needed when comparing alamar blue reduction of different cell types, and errors may arise when extrapolating the curves obtained by one cell type to another.

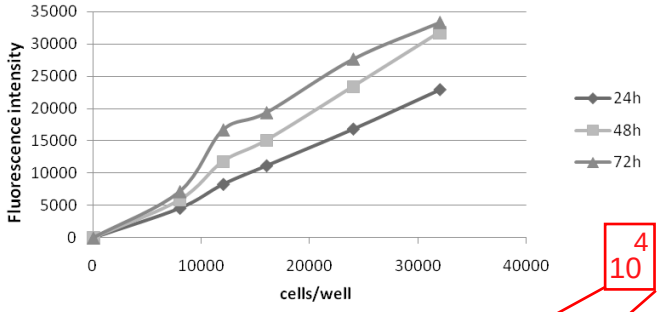

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Fig. 1. Standard curve of alamar blue reduction versus cell concentrations. ASZ001cells were cultured at concentrations between 1x104 and 4x104 cells/well. The number of viable cells correlated linearly with the magnitude of dye reduction at 24h and 48 h. The values of alamar blue reduction were corrected for background values of negative controls containing medium without cells. Data are presented as means

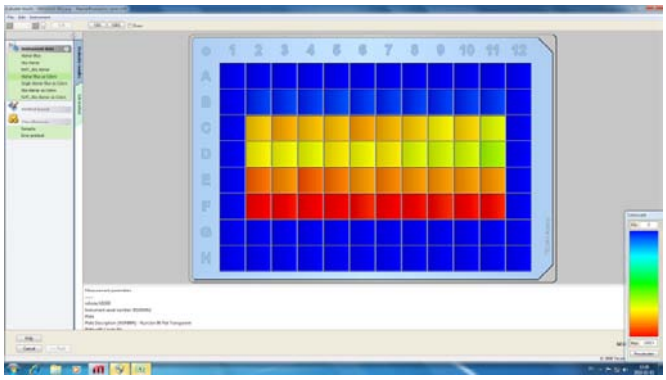

Fig. 2. HaCaT: B changed to medium with serum, C changed to medium with serum, D medium with serum without cells, E serum free medium, F serum free medium + IGF-1 (50 ng/ml)

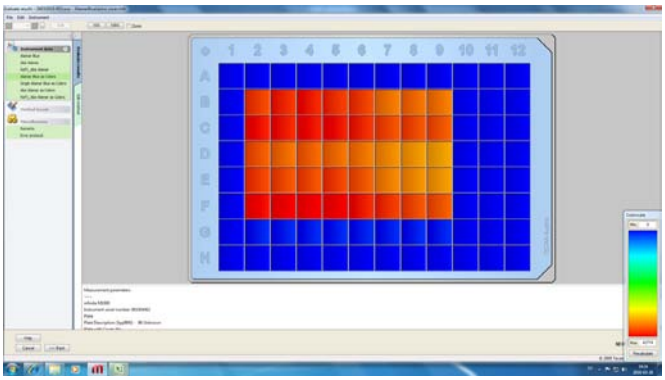

Fig. 3. ASZ001: B - changed to medium with serum, C - changed to medium with serum, D - only medium without cells, E - only serum without cells, F - serum free medium, G - serum free medium + IGF-1(50 ng/µl)

On the basis of these results we decided for the following experiments to use a cell concentration of 1 x 10^4 cells/well and an incubation time with alamar blue of 4 h.

Effect of IGF-IR stimulation of growth of basal cell carcinoma cells.

Alamar Blue assay for cell viability was performed to determine the effect of IGF1 treatment. After spliting into 96-well plates at a concentration of 1x10^4 cells/well the cells were allowed to attach and grow for 24 hours. Then they were washed three times with PBS, and serum-starved for 12 hours in order to induce sincronization of cell cycle. We had four conditions, which represented: 1) the background values of negative controls containing medium without cells, 2) no supplement (CTL) – serum-starved cells, 3) supplement with serum, 4) stimulated with IGFI (50 ng/mL) in the absence of serum for 12 hours. Alamar blue was added with 4 hours before reading. Cell viability was confirmed by fluorescence intensity which was measured using microplate reader Infinite M1000 (Figure 2, Figure 3).

The fluorescence intensity was offered by Tecan's universal data analysis - Magellan software. Means of fluorescence intensity are shown (Table I). The experiment was repeated three times with similar results.

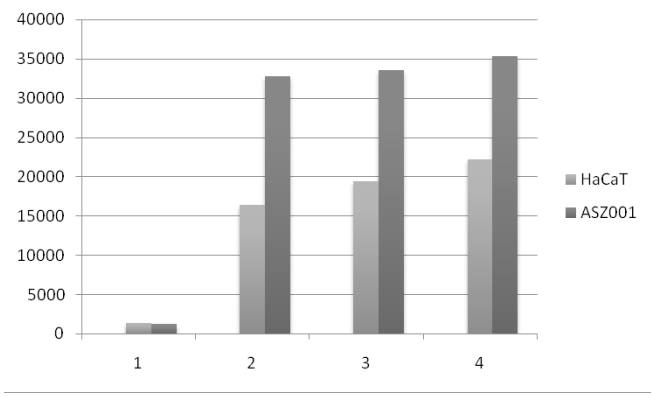

Fig. 4. The effect of IGF1 on growth of HaCaT and basal cell carcinoma cells. The cell lines were cultured in 96-well plates. The cells were allowed to attach and grow for 24 hours. They were then serum-starved for 12 hours, and then 2) no supplement (CTL), 3) supplement with serum or 4) stimulated with IGFI (50 ng/mL) in the absence of serum for 12 hours. 1) represent the background values of negative controls containing medium without cells. Means of fluorescence intensity are shown. The experiment was repeated thrice with similar results.

The viability of HaCaT and ASZ001 cells was tested both in the presence and in the absence of IGF1. As can be seen, IGF-I was the effective stimulant and caused more significant growth response in mouse basal cell carcinoma ASZ001 cell line than in HaCaT cells. These data are well consistent with the growth stimulatory effects of IGF-I. (Figure 4).

Discussion

Alamar Blue is a water-soluble dye that has been previously used for quantifying in vitro viability of various cells [37,40]. Due to the fact that it is extremely stable and more importantly nontoxic to the cells, continuous monitoring of cultures over time is possible37. Mainly for this reason, this test has been considered superior to classical tests for cell viability such as the MTT test [41,42]. However, the MTT test necessitates killing the cells, making it impossible to follow-up cell cultures.

ASZ001 cell lines have been useful as models for studying various functions basal cell carcinoma cells. In this study, we report on the applicability of alamar blue assay for quantifying cell numbers and viability of HaCaT and ASZ001 mouse basal cell carcinoma cells.

Addition of alamar blue to cultured cells does not alter their viability, unlike that which occurs during monitoring by Trypan Blue exclusion [37,39]. We confirmed the viability of the cells subjected to alamar blue using microplate reader Infinite M1000. IGF1 has been reported to increase

Table I. The effect of IGF-1 on growth of HaCaT and ASZ001 basal cell carcinoma cells

Crt No.	Conditions	Mean fluorescence intensity(nm) (HaCaT)	Mean fluorescence intensity(nm) (ASZ001)
1	Medium with serum without cells	1352.3	1277.9
2	Changed to serum free medium	16444	32745
3	Changed to medium with serum	19398	33534
4	Changed to SFM+IGF1(50ng/ml)	22203	35309

proliferation of HaCaT and basal cell carcinoma cells. We found in IGF-1 treated cells, a increase alamar blue reduction compared with control cells suggesting a certain degree of stimulatory response in these cells. This finding could be better explained by the higher sensitivity of the alamar blue assay in detecting modest differences in cell number. The high expression of IGF–1R in neoplastic cells and tissues combined with its crucial roles in cancer cell growth is making this tyrosine receptor an attractive target for anticancer treatment. So far, numerous attempts to directly inhibit IGF–1R functions caused massive apoptosis of tumor cells in vitro and in vivo, inhibition of tumorigenesis [19,43,44,45,46,47,48] and metastases [49,50]. Overall, strategies leading to downregulation of the receptor have been associated with the strongest antitumor efficacies51.

Conclusion

Taken together, our data suggest that IGF-IR may be involved in the growth of basal cell carcinoma and points to the possibility of the involvement of IGF-IR inhibitors as a treatment modality. Further studies on larger collections of cases are needed to evaluate the expression level and distribution of IGF-IR and its correlation to different histopahological subtypes.

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