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Histological evaluation of scar tissue inflammatory response: the role of hGH in diabetic rats

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Summary. This paper describes a polymer site-specific delivery system containing human growth hormone in an *in vivo* model of scarring in the diabetic state.

Copolymer discs with the hormone were introduced into incisions made in rats previously injected with streptozotocin in order to induce diabetes. Tissue specimens for evaluation were obtained at 3, 7 or 10 days after the procedure. Study groups were healthy rats and diabetic rats untreated or treated with/without the hormone. Histological sections were prepared for light microscopy examination of wound zones.

Three and 7 days after surgery, polymer remains could be observed in the subcutaneous tissue. These remnants induced a moderate foreign body reaction. The number of macrophages detected was directly related to neovessel formation and metalloelastase expression. The $CD4^+/CD8^+$ ratio was low during the initial follow up stages (3 and 7 days) in untreated diabetic rats, yet an increased ratio corresponding to areas around the polymer remains was noted in the animals treated with copolymer loaded with the growth hormone.

Copolymer is biodegradable *in vivo* and may be used as a vehicle for the slow release of active substances. The presence of the hormone at the site of skin injury induces cell proliferation and enhances the repair process.

Key words: Diabetes, Drug delivery, Growth factor, Immune response, Wound healing

Introduction

In clinical situations of deficient scarring, as occurs in skin burns or in the diabetic state (Bitar, 1997), the administration of human growth hormone (hGH) improves the regeneration capacity of the skin (Massey et al., 1998; Gimeno et al., 2003), reducing the scarring time (Herndon et al., 1990; Barret et al., 1999). Given that several types of growth factors can modulate wound healing in tissue repair processes, controlled drug delivery systems could prove to be useful tools for their administration.

Controlled release systems can efficiently deliver hormones and growth factors to target tissues acting as biodegradable vehicles with the capacity to gradually release their contents. There are many examples of such systems in the literature (collagen sponges, gels, creams, liposomes, different polymers) (Agren, 1998; Berrocal et al., 1998; Ismail et al., 2000; Vlachou et al., 2000).

Herein, we assess the *in vivo* effects of hGH administered using a new copolymer comprised of 2-hydroxyethylmethacrylate (HEMA) and polyvinyl pyrrolidone (PVP) as the vehicle. Due to their hydrophilic nature, both these substances have been extensively used in ophthalmological and pharmaceutical preparations as degradable biomaterials. Their tolerance, biocompatibility and lack of toxicity in biological systems have been amply described both *in vitro* (De Groot et al., 2001; Wiese et al., 2001) and *in vivo* (Mokry et al., 2000; Cadee et al., 2001; Piza-Katzer et al., 2002).

In normal conditions, the repair of injured tissue involves an inflammatory process, in which white blood cells (Boyce et al., 2001) act in the first instance. This initial reaction is followed by fibroblast proliferation and extracellular matrix remodelling by tissue factors (Clark et al., 1997) and matrix metalloproteinases (MMPs), respectively. In pathological settings such as diabetes, macrophage infiltration, cell proliferation (Galeano et al., 2001) and angiogenesis (Altavilla et al., 2001) are delayed. Furthermore, metalloproteinase expression occurs earlier and leads to altered collagen deposition (Sato et al., 1999), since these enzymes play an essential role in connective tissue degradation during the development of certain diseases.

Our study was designed to evaluate the response

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shown by white blood cells and extracellular matrix proteins in a skin wound repair model established in diabetic rats treated with a copolymer loaded with hGH to achieve the controlled, slow release of the hormone.

Materials and methods

Animals

The experimental animal used was the male Wistar rat of 150 g approximate weight. Diabetes was induced in 54 healthy rats (6 per treatment group and study time) by two intraperitoneal injections of streptozotocin (700 µg/Kg) (Andreassen and Oxlund, 1987). A further 10 untreated healthy rats served as the control group. Over a 30-day period, the animals were subjected to weekly determinations of glycaemia in urine by using Multistix® Reagent Strips (Multistix® 10 SG, Bayer Corporation, USA), to check that the diabetes had not reversed. This was followed by making 3 cm incision on the back of each animal, and a copolymer disc, 300 mm thick and 10 mm in diameter containing hGH, prepared as described elsewhere (Gimeno et al., 2002), was then introduced through the incision and placed over the fascia and in contact with the wound edges. The skin was closed over the wound by stapling. Animals were sacrificed 3, 7 and 10 days after surgery and tissue specimens were obtained for evaluation. Four study groups were established according to whether the animals were non-diabetic (control, n=10), diabetic and untreated (DR, n=18), diabetic and treated with the polymer alone (DRP, n=18) or diabetic and treated with the polymer containing hGH (DRPGH, n=18).

Histological study

Tissue samples were taken by reoperating the animals to remove the wound zone including the incision and surrounding tissue. For light microscopy, skin fragments were fixed by immersion in Bouin solution, embedded in paraffin and cut into 5 m transverse sections using a microtome (Microm, Barcelona, Spain). The sections were then stained using haematoxylin eosin and Masson's trichrome stain.

Immunohistochemical study

The following monoclonal antibodies were used to identify cells and proteins: anti rat monocytes /macrophages (ED1, Serotec, Oxford, UK), anti-$CD4^+$ and anti $CD8^+$ (Labgen, Barcelona, Spain), anti-α-actin (clone 1A4, Sigma, Spain) and anti-macrophage metalloelastase or MMP-12 (R&D Systems, Abingdon, UK). The alkaline phosphatase avidin-biotin labelling technique was used to detect the antigen-antibody reaction. This protocol involves incubating tissue specimens with the primary antibody diluted in phosphate buffered saline (PBS) overnight at 4 °C, followed by incubation with IgG-biotin (1:400 in PBS) for 45 min at room temperature, and labelling with avidin coupled to alkaline-phosphatase (1:200 in PBS) for 45 min at room temperature. Immunocomplexes were developed using the Vector, red alkaline phosphase substrate kit (Vector Laboratories Inc., Burlingame, USA). Nuclei were counterstained for 15 min with haematoxylin.

Statistical analysis

Data corresponding to epidermal thickness, inflammatory cell proportions (ED1, $CD4^+$ and $CD8^+$), extent of neoangiogenesis and degradative enzyme expression (MMP-12) were statistically analysed by one-way ANOVA and the Student-Newman-Keul's test to establish significant differences between the study groups. The data are provided as means ± standard deviation.

Results

Inflammatory cell involvement

Using specific antibodies we were able to evaluate the presence of blood vessels, macrophages and the lymphocyte ratio $CD4^+$:$CD8^+$ in the wounds produced. These histological tests indicated that the polymer induced a weak foreign body reaction, that the inflammatory response to damage in the diabetic animals was delayed and that the hGH-loaded polymer induced

Table 1. Post-surgery inflammatory cells at the wound area.

	3 DAYS		7 DAYS		10 DAYS	
	$ED1^+$ (%)	CD4/CD8	$ED1^+$ (%)	CD4/CD8	$ED1^+$ (%)	CD4/CD8
NR	3.68±1.55	54	4.23±1.87	12.5	3.68±1.23	2.5
DR	0±0	0	0.52±0.18	21	1.23±0.44	5.9
DRP	0±0	0	0.46±0.26	23.3	1.14±0.67	6.7
DRPGH	7.11±5.25	72	4.42±2.3	18.4	3.35±1.48	4.9

NR: non-diabetic group (control); DR: diabetic and untreated; DRP: diabetic and treated with the polymer alone; DRPGH: diabetic and treated with the polymer containing hGH.

an accelerated tissue and cell response similar to that observed in the control non-diabetic animals (Table 1).

At the wound site, diabetic animals treated with hGH showed a greater extent of neoangiogenesis comparable to that observed in the wounds of non-diabetic controls. In the DR group, neoangiogenesis was delayed, with areas of α-actin positive vessels appearing in the depths of the dermis at 7 days post-surgery (Fig. 1). In the presence of adipose granulation tissue, an increased extent of angiogenesis associated with the presence of many inflammatory cells was noted.

The $CD4^+$:$CD8^+$ ratio increased in 3 day-old wounds in the control and DRPGH groups due to raised $CD4^+$ lymphocyte levels. The presence of this cell type at the first follow-up time in the hGH-treated wounds diminished during the course of healing.

In the DR and DRP groups, lymphocyte action was delayed with respect to that observed in DRPGH. Peak macrophage and $CD4^+$ levels could be seen at the site of incision and around the polymer remnants at 7 days post-surgery. $CD8^+$cells, which were minimal throughout the experiment, were always found close to

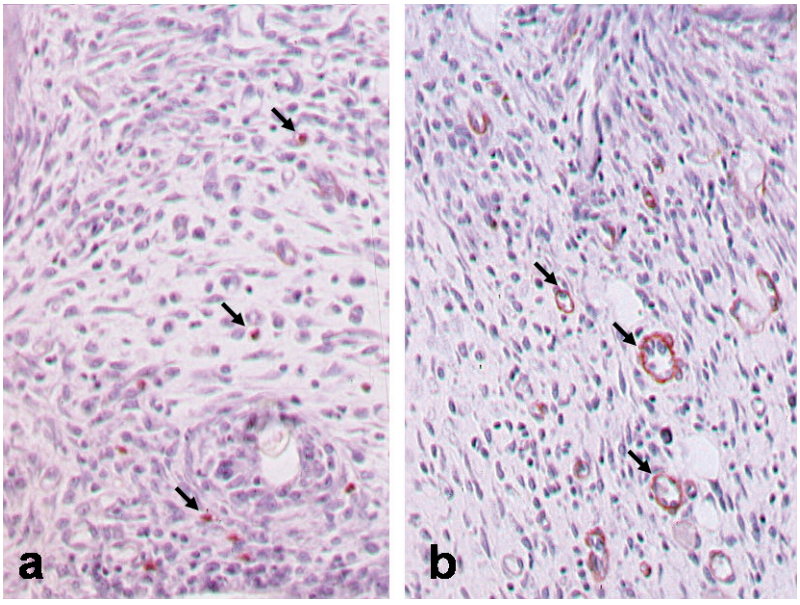

Fig. 1. The effect of growth hormone was to increase the rate of vascularisation, a decrease vascularisation with time, using immunohistochemistry for α-actin, was observed in specimens for DRP **(a)** with respect to that observed in DRPGH **(b)**. Positive cells (arrows). MO, x 400

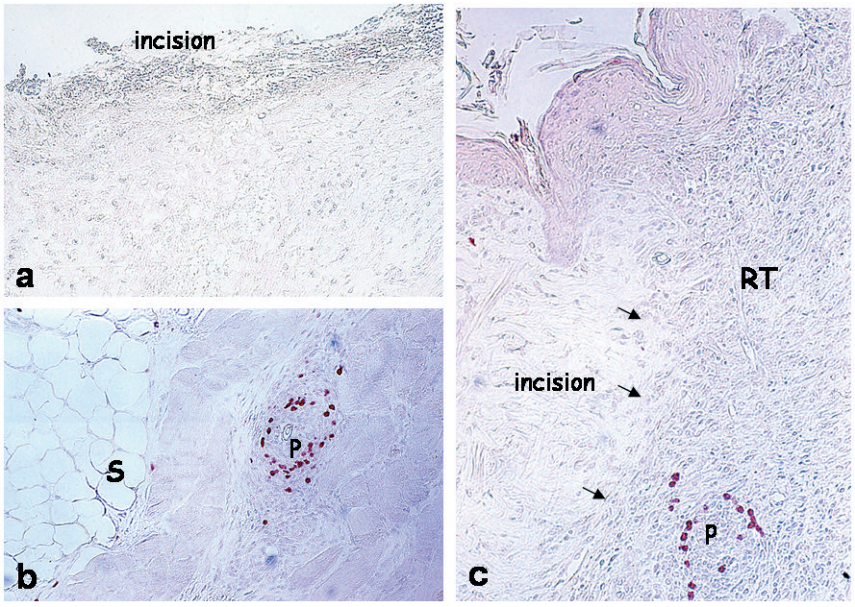

Fig. 2. The expression of MMP-12 was delayed in DR non hGH treated. Immunolocalisation of MMP-12 showed the presence of positive cells (in red) surrounding polymer remains. **(a)** DRP 3 days, **(b)** DRPGH 3 days and **(c)** DRPGH 7 days. P: polymer; S: Seroma; RT: Remodeling tissue: Incision. MO, x 200

polymer remains.

Metalloproteinase expression

Staining for MMP-12 (Fig. 2), a macrophage metalloelastase that degrades a large component of the extracellular matrix, revealed the expression of this enzyme in the vicinity of remnants of the polymer remnants or in the deep dermis of the incision wounds. The appearance of MMP-12 was delayed in DR and DRP when compared to controls or DRPGH. ED1(indicating monocytes/macrophages) showed a similar expression pattern.

Discussion

The VPH polymer system is able to transport and release agents that stimulate tissue regeneration at the site of injury. Having already established the efficiency of this system in terms of its effects on cell growth *in vitro* (Gimeno et al., 2002, 2003), we decided to evaluate its ability to deliver hGH in an *in vivo* model in an effort to improve the wound repair process in a model of diabetes in the rat.

Our observations indicate that the polymer was well tolerated by the recipient animal and that the controlled, slow release of hGH improved wound healing in diabetic animals. In diabetic rats (DR), scarring was delayed with respect to the normal scarring shown by control animals and treatment with hGH was found to normalise the situation, such that the scarring time was the same for the diabetic animals treated with the hormone and the control non-diabetic non-treated animals.

The appearance of white blood cells at the site of injury is a key factor affecting the scarring process. It has been demonstrated that $CD4^+$ lymphocytes activate the scarring response (Wojciak and Crossan, 1994) and that $CD8^+$ lymphocytes delay the response (Barbul et al., 1989) to the extent that the $CD4^+$:$CD8^+$ ratio reflects the tissue repair process. It has also been reported that the presence of macrophages promotes the formation of new blood vessels (Polverini and Leibovick, 1985) and extracellular matrix components such as fibronectin and MMPs (Brown et al., 1993; Salo et al., 1994).

The foreign body reaction induced by the polymer used was moderate, at the initial stages of tissue repair, the $CD4^+$:$CD8^+$ ratio was highest in diabetic animals treated with the hGH-loaded polymer, with ratios approaching control values and always above those recorded in the DR and DRP groups. Boyce's group (Boyce et al., 2001) similarly attributed a faster repair process to the $CD4^+$ lymphocyte subset during an evaluation of hypertrophic processes in keloid scars.

The metalloproteinase MMP-12 is synthesised by macrophages and its appearance thus coincided with the presence of marcrophages at the repair site. As described by Vaalamo (Vaalamo et al., 1999) this enzyme promotes the migration of these cells to the area of tissue damage.

The presence of the polymer in the deep layers of the dermis induces a discrete foreign body reaction around the polymer remains. This reaction is associated with fibrosis which, along with the action of the growth hormone, first induces the neoformation of extracellular matrix and secondly promotes cell proliferation. Both these events hasten scar closure and therefore reduce the risk of complications related to defective scarring in animals with diabetes.

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