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Treatment of skin ulcers with adenylate deaminase, a glycoprotein from microscopic fungus *Penicillium lanoso-viride*

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Abstract

The effect of topical application of adenylate deaminase (AMPD), an immunomodulatory glycoprotein produced by the microscopic fungus *Penicillium lanoso-viride*, on the experimental skin ulcers was studied in rats. In total, 28 animals in four groups were used. Carbopol hydrogel containing AMPD in doses of both 3.0 and 0.3 U mL^{-1} accelerated healing of the ulcer ($p < 0.05$). Complete (100 %) epithelialisation on the 21st day was observed in a group of animals treated with AMPD 3.0 U mL^{-1} and partial (60 %) epithelialisation in the group treated with AMPD, 0.3 U mL^{-1}. No animal showed entired epithelialisation of skin ulcer treated with Carbopol and untreated control groups. The morphological results were supported by histological findings. Standard blood chemistry and complete blood counts parameters were within the normal range and without significant differences. Experimental results indicated that locally administered glycoprotein-adenylate deaminase hydrogel possesses ulcer healing activity.

Key words: adenylate deaminase, healing, microscopic fungi, *Penicillium lanoso-viride*, skin ulcer.

Introduction

Wound healing is a dynamic, interactive process involving soluble mediators, blood cells, extracellular matrix, and parenchymal cells. The healing has three phases – inflammation, tissue formation, and tissue remodeling, which overlap in time (Singer, Clark 1999).

At the cellular level, each phase is directed by the coordinated interaction of several types of cells, including inflammatory cells and native skin cells such as fibroblasts, keratinocytes and vascular endothelial cells. At the molecular level, cytokines regulate inflammatory cells during the early stages of wound healing, and throughout the process polypeptide growth factors play dominant roles in the regulation of the proliferation and differentiation of native skin cells and the synthesis of extracellular matrix (Martin 1997; Goldman 2004). It is known that various growth factors, cytokines, and chemokines function in the wound-healing process (Werner, Grose 2003). Platelets, neutrophils, macrophages, endothelium, fibroblasts and epithelium express and release epidermal

growth factor, transforming growth factors, platelet-derived growth factor, interleukins, proteases, matrix proteins and other compounds (Parenteau, Hardin-Young 2007). The regulation of the temporal and spatial expression of growth factors is of major significance for normal repair (Werner, Grose 2003).

Numerous experiments have been carried out to investigate the pharmacological potential of different growth factors and other mediators. Unfortunately, the overall experience with application of these agents to accelerate wound healing has been discouraging. This is not surprising, considering that wound repair is the result of a complex set of interactions among soluble cytokines, formed blood elements, extracellular matrix, and cells (Pierce, Mustoe 1995; Singer, Clark 1999; Bello, Phillips 2000). Nevertheless, intensive research in wound healing is continuing. Current research is leading to new therapies that can be divided into the following classes: growth factors, skin substitutes, extracellular matrix proteins, stem cell therapy, gene therapy, protease inhibitors, angiogenesis stimulants, nitric oxide-releasing agents, adenosine agonists, immunostimulants, vasoactive compounds and granulating agents (Petrova, Edmonds 2006).

Non-specific adenylate deaminase (AMP aminohydrolase, EC 3.5.4.6; AMPD) is a glycoprotein (g_M 210 kD) produced by microscopic fungus *Penicillium lanoso-viride* during a particular phase of growth, i.e., conidiospore formation (Revelina et al. 1981). *In vivo* studies on experimental animals have demonstrated that purified AMPD has diverse immunomodulating properties that influence both cell-mediated and humoral immunity reactions. Injections of AMPD have been shown to augment the formation of antibody-producing cells in spleen, facilitate lymphocyte proliferation *in vitro*, activate mice peritoneal macrophages (Nikolajeva et al. 1999), stimulate natural killer cell and tumour adhesion, restrict growth of several tumours (Zak et al. 1986), enhance host resistance to infectious agents (Nikolajeva et al. 1996), and inhibit experimental autoimmune encephalomyelitis (Nikolajeva et al. 2000). On the basis of previous investigations on the influence of AMPD on the systemic immune response, we hypothesized that AMPD could contribute also the local immune processes in the skin.

The present investigations were designed to evaluate the influence of topically administered AMPD produced by microscopic fungus *Penicillium lanoso-viride* on healing of skin ulcer in rats.

Materials and methods

Animals and animal care

Male Wistar rats (123 to 169 g) were obtained from the laboratory of experimental animals of the Riga Stradins University. The rats were housed in a room with controlled environment (20 ± 2 °C, 55 ± 3 % relative humidity, ventilation and a 12 h alternating light-dark cycle). Standard pelleted complete diet and water were supplied *ad libitum*.

The study was carried out at the University of Latvia, Latvia. The protocol of study was reviewed and approved by the Animal Ethics Committee at the Food and Veterinary service Republic of Latvia.

Materials

AMPD that was sterile and free of endotoxin was prepared and purified in our laboratory

(Nikolajeva et al. 1996). One unit (I U) of enzyme activity was determined as the quantity of AMPD that deaminated 1.0 μmol of 5`-AMP to 5`-inosine monophosphate per min at 37 °C, pH 6.0. Specific activity of AMPD was 10 U per mg protein. Carbopol 940 was obtained from PharmaZell GmbH (Germany). All other chemicals were at pharmaceutical or analytical grade.

Dressings

The polymer-based bioadhesive hydrogel was generated from individual constituents, and finished composition contained 1 % Carbopol 940, 1.35 % triethanolamine, 0.9 % sterile NaCl and filter-sterilized AMPD used in two dosages 0.3 U mL^{-1} or 3.0 U mL^{-1}. The Carbopol gel without active components did not contain AMPD. Gels were prepared before the beginning of the experiment, and complete compositions were maintained in a refrigerator (4 ± 2 °C).

Measurement of healing activity

An experimental skin ulcer was developed under light ether anaesthesia by hypodermic injection of 0.5 mL 9 % acetic acid in the right foreleg and intraperitoneal injection of 6 % dextran in a dose of 300 mg kg^{-1}. Necrosis of the skin and a skin ulcer developed within three days.

The animals were divided into four groups of six to eight each. Animals of group 1 were treated with the Carbopol gel containing AMPD 3.0 U mL^{-1}. Animals of group 2 were treated with Carbopol gel containing AMPD 0.3 U mL^{-1}. The group 3 animals served as reference standard and treated with Carbopol gel without AMPD. The group 4 animals were left untreated and formed the control. The gels were topically applied to the wound bed once a day beginning with the 4th day following the initial wounding (hypodermic injection).

The following parameters were estinated: ulcer closure rate, time of epithelialisation and feeling of pain. The ulcer closure rate was assessed by planimetric measurement of the wound area. The measurements were taken in the beginning of treatment on the 4th day, and also on the 7th, 14th and 21st day. Ulcer closure (V) as a percentage was calculated as follows (Kirker et al. 2002): $V = (A_0 - A_t) / A_0 \times 100$, where A_0 is the original ulcer area and A_t is the area of ulcer on the day of measurement. The process of epithelialisation was evaluated by the coefficient of regeneration (R_{coef}) and regression index of regeneration (R_{reg}):

R_{coef} = the initial area of the ulcer (mm^2) / the area of the ulcer on the day of measurement (mm^2);

R_{reg} = R_{coef} in the experimental group / R_{coef} in the control group.

The period of epithelialisation was calculated as the number of days required for dead tissue remnants to fall and elimination of residual raw ulcer. The feeling of pain was evaluated by light touching with a wood-pointer to the wounded region.

Blood tests

Blood tests were taken from sublingual vein in three to four animals from each group on the 14th and 21st day. The following biochemical and blood counts parameters were estimated – white blood cell count, red blood cell count, haemoglobin, hematocrit, platelet count, leukocyte subpopulation differential, alanine aminotransferase, aspartate

aminotransferase, albumin, alkaline phosphatase, cholesterol and total protein. The percentage of albumin, alpha 1, alpha 2, beta 1, beta 2 and gamma proteins was calculated by protein electrophoresis in PAAG. Blood tests were carried out with a Beckman Coulter (USA) and ILab-300 Plus (Instrumentation Laboratory, USA).

Pathomorphological and histological studies

Three to four animals from every group were sacrificed on the 14th and 21st day. Skin tissue samples (2 × 2-cm pieces) were collected while the animal was fully anesthetized. The skin was excised from the central regions of the ulcer. After tissue harvesting, animals were euthanatized. Samples were fixed in 10 % neutral buffered formalin, dehydrated and then embedded in paraffin. Tissue sections (2 to 4 μm in cross-section) were stained by routine procedures: hematoxylin and eosin for the evaluation of leukocyte infiltration, cellular debris and scar formation, and by van Gieson's connective tissue staining (Böck 1989). The main features were estimated by a scoring: – absent; + weak; ++ medium; +++ strong.

The weight of heart, kidney, liver, testes and epinephric glands was estimated, and their pathomorphological investigation was carried out..

Statistics

All values were expressed as the means ± standard deviations (S.D.). The differences between groups were analysed by one-way ANOVA test from summary data. Values of p less than 0.05 were considered to be significant. The experiment was conducted in duplicate for confirmation.

Results

Ulcer closure in rats treated with adenylate deaminase-containing gels

Hypodermic injection of solution of acetic acid resulted in the necrosis of the skin, and a skin ulcer developed in three days. Total open circular wound surface area reached its peak on the 4th day with an average of 219 ± 78 mm^2.

The rate of ulcer closure in both AMPD gels treated groups was significantly ($p < 0.05$) higher on the 14th and 21st day, as compared to that of the Carbopol and Control groups (Fig. 1). Moreover, the lowest dose of AMPD (0.3 U mL^{-1}) showed a significant increase ($p < 0.05$) in the ulcer closure rate already on the 7th day after three applications. There was a small decrease of the rate of closure at this dose in the subsequent week, while there was a significant increase at the higher dose of 3.0 U mL^{-1}. The rate of the closure decreased in both groups in the third week but there still remained differences in the closed area in comparison with the Carbopol and control groups. There was no significant difference ($p > 0.05$) in the rate of ulcer closure between Carbopol and control groups.

The estimated epithelialisation time corresponded to the closure rate. There was complete (100 %) epithelialisation on the 21st day in the group of animals treated with AMPD 3.0 U mL^{-1} and epithelialisation in 60 % of animals receiving AMPD 0.3 U mL^{-1} (Fig. 2). No animal showed complete epithelialisation of cutaneous ulcer in Carbopol and control groups.

The epithelialisation process evaluated by R_{coef} (Fig. 3) and R_{reg} (Fig. 4) confirmed that AMPD improves healing in a dose dependent manner. Both coefficients were higher in the group treated with AMPD 0.3 U mL^{-1} during first two weeks but the larger dose showed

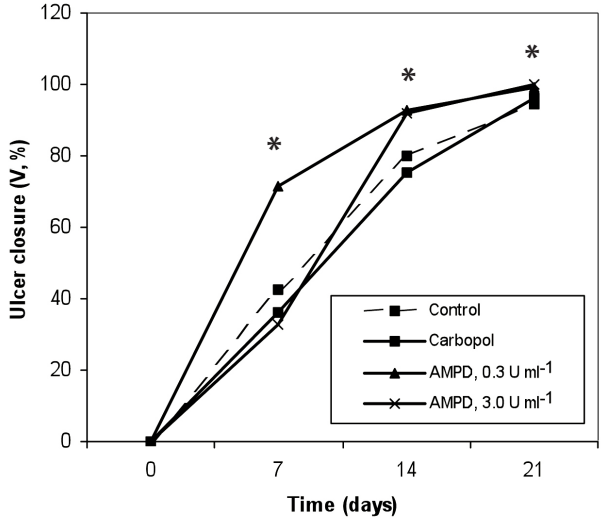

Fig. 1. Influence of AMPD on the ulcer closure (V). Experimental skin ulcers of rats were treated with AMPD 0.3 U mL^{-1}, AMPD 3.0 U mL^{-1}, Carbopol without AMPD, or left untreated (Control). Gels were topically applied to the ulcers once a day beginning with the 4th day following the initial wounding (hypodermic injection). Percent of ulcer closure (V) was calculated as follows: $V = (A_0 - A_t) / A_0 \times 100$, where A_0 is the initial area of the ulcer and A_t is the area of ulcer on the day of measurement. Replication on days 7 and 14 was six to eight animals and on day 21 – three to four animals. *, significant difference ($p < 0.05$) when compared with the Carbopol and Control groups.

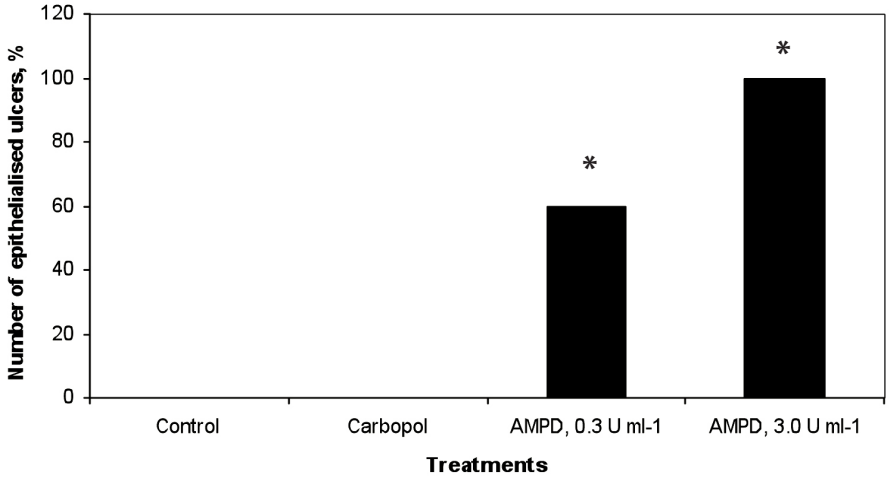

Fig. 2. Percentage of number of epithelialised ulcers at the end of experiment on the 21st day following wounding. Experimental skin ulcers of rats were treated with AMPD 0.3 U mL^{-1}, AMPD 3.0 U mL^{-1}, Carbopol without AMPD, or left untreated (Control). Gels were topically applied to the ulcers once a day beginning with the 4th day following the initial wounding (hypodermic injection). Replication was three to four animals in each group were. The experiment was conducted in duplicate. *, significant difference ($p < 0.05$) when compared with the Carbopol and Control.

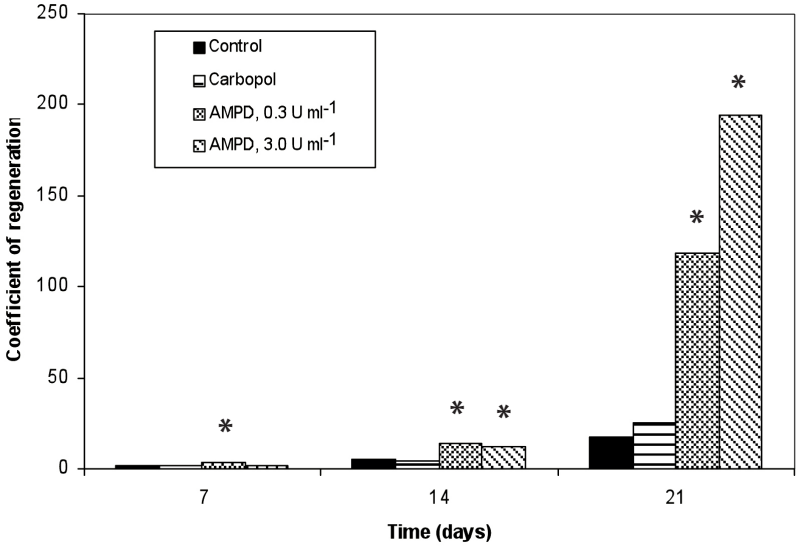

Fig. 3. Epithelialisation expressed as coefficient of regeneration (R_{coef}) in animal groups with different treatment of ulcers. Experimental skin ulcers of rats were treated with AMPD 0.3 U mL^{-1}, AMPD 3.0 U mL^{-1}, Carbopol without AMPD, or left untreated (Control). Gels were topically applied to the ulcers once a day beginning with the 4th day following the initial wounding (hypodermic injection). R_{coef} was calculated by dividing the initial area of the ulcer (mm^2) by the area of the ulcer on the day of measurement (mm^2). *, significant difference ($p < 0.05$) when compared with the Carbopol and Control.

better results later in the third week of application.

Effects of adenylate deaminase on the parameters of blood tests and general health

To establish that no bias mediated via infection or inflammation occurred, standard blood chemistries and complete blood counts were carried out. All blood counts parameters measured (white blood cell count, red blood cell count, haemoglobin, hematocrit, platelet count, leukocyte subpopulation differential) were within the normal range (data not shown). Similarly, all blood chemistry parameters measured (alanine aminotransferase, aspartate aminotransferase, albumin, alkaline phosphatase, cholesterol, total protein) were within the normal range (data not shown). No significant differences ($p > 0.05$) between animal groups were found during the course of experiment.

All animals had a healthy course, with no clinical complications or deaths in either group. Additionally, all animals exhibited normal posture and appetite during the post-injury period. The increase of rat weight during the experiment showed a general well being of experimental animals, similar ($p > 0.05$) among treatment groups (data not shown).

Pathomorphological and histological findings

Ulcer healing differed in intensity and stages of re-epithelialisation, as well as development of granulation and connective tissue in the examined histological specimens.

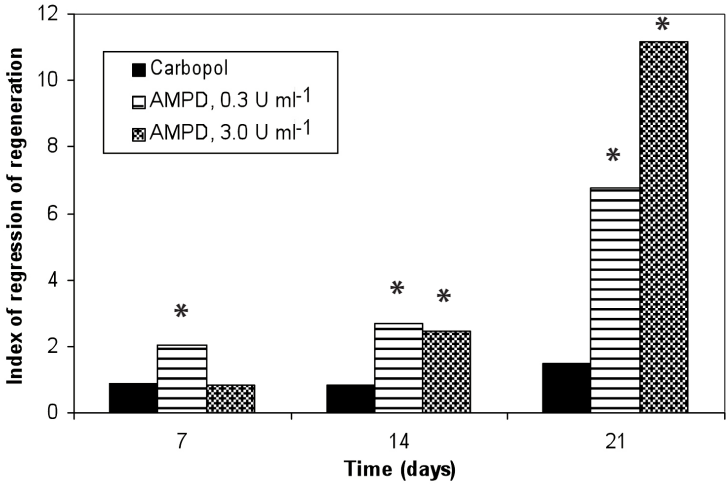

Fig. 4. Influence of AMPD and Carbopol on the epithelialisation of skin ulcers expressed as index of regression of regeneration (R_{reg}). Experimental skin ulcers of rats were treated with AMPD 0.3 U mL^{-1}, AMPD 3.0 U mL^{-1}, Carbopol without AMPD, or left untreated (Control). Gels were topically applied to the ulcers once a day beginning with the 4th day following the initial wounding (hypodermic injection). R_{reg} was calculated by dividing the coefficient of regression (R_{coef}) in the experimental group by R_{coef} of the control group (Fig. 3). *, significant difference ($p < 0.05$) when compared with the Carbopol and Control.

A strong infiltration of leukocytes was observed in the skin samples from the AMPD 0.3 U mL^{-1} and the Carbopol group on the 14th day, and a moderate infiltration in the AMPD 3.0 U mL^{-1} group (Table 1). There was also a significant amount of cellular debris: small amount in the group treated with AMPD 3.0 U mL^{-1}, moderate in the group of AMPD 0.3 U mL^{-1}, and strong in the Carbopol group. Histological estimation of epithelialisation confirmed planimetric measurement of the ulcer area. The dermis was covered with a fibrin clot what was infiltrated by inflammatory cells and fibroblasts. Connective tissue formation was evaluated as medium score in the AMPD 0.3 U mL^{-1} and Carbopol group, and with a strong score in the AMPD 3.0 U mL^{-1} group. There was a strong similarity between animals regarding features of the scars.

The amount of leukocytes and cellular debris in the region of ulcer decreased during the process of healing until the end of experiment on the 21st day, more or less in all treatment groups, possibly with exception of untreated control (Table 1). It was noted that epithelialisation was completed in the AMPD 3.0 U mL^{-1} group. Notable reduction of scar in comparison with the 14th day was observed only in the AMPD groups in particularly in the of the AMPD 3.0 U mL^{-1} group. The ulcer region revealed a structured type of dermis with ripening connective tissue, parallel to the surface arranged bundles of collagen and full-blooded capillaries, and did not visibly differ between groups.

Heart, kidney, liver, testes and epinephric glands, both on the 14th, and on the 21st day, showed normal appearance and structure without any shift from the norm including weight of organs (data not shown). There was no significant difference between groups of animals ($p > 0.05$).

Table 1. Effect of AMPD on the histological parameters of skin ulcer regions. Experimental skin ulcers of rats were treated with AMPD 0.3 U mL^{-1}, AMPD 3.0 U mL^{-1}, Carbopol without AMPD, or left untreated (Control). Gels were topically applied to the ulcers once a day beginning with the 4th day following the initial wounding (hypodermic injection). Skin tissue samples were excised from the central regions of the ulcer on the 14th and 21st day, processed and stained by hematoxylin and eosin for the evaluation of leukocyte infiltration, cellular debris and scar formation, and by van Gieson's connective tissue staining. The features were expressed by scoring: – absent; + weak; ++ medium; +++ strong. N – not determined

Parameter	Treatment			
	Control	Carbopol	AMPD (0.3 U mL^{-1})	AMPD (3.0 U mL^{-1})
14th day				
Number of animals	0	4	4	3
Epithelialisation	N	+	+++	++
Cellular debris	N	+++	++	+
Connective tissue	N	++	++	+++
Leukocytes	N	+++	+++	++
Scar	N	+++	+++	+++
21st day				
Number of animals	3	3	3	3
Epithelialisation	++	++	+++	+++
Cellular debris	+++	++	++	–
Connective tissue	+++	+++	+++	+++
Leukocytes	+++	++	–	+
Scar	+++	+++	++	+

Discussion

The two main objectives of pharmacology of wound healing are to determine the influence of various measures in wound management and to screen drugs that promote healing. Several materials have been used so far and are reported to affect healing differently. In the present study, we have shown that AMPD, a fungal glycoprotein enzyme, increased the healing rate of skin ulcers, compared with that of untreated ulcer or carrier hydrogel treatment. Although healthy young rats are known to heal skin wounds efficiently and there is likely little room for significant improvement, ulcer closure was significantly promoted in both AMPD hydrogel-treated groups (Fig. 1, 2). The morphological results were supported by histological findings (Table 1).

Acute wounds normally heal in a very orderly and efficient manner by a highly controlled repair process requiring numerous cell-signalling events (Diegelmann, Evans 2004). The initial response to a cutaneous wound induces powerful transcriptional activation of pro-inflammatory stimuli what may alert the host defence. Subsequently, and in the absence of infection, inflammation subsides and is replaced by angiogenesis and remodeling (Deonarine et al. 2007). In our experiments, a greater amount of inflammatory cells was observed in superficial and deep areas of the granulation tissue of the control

groups compared to the AMPD groups, both on the 14th and on the 21st day following wounding. Despite the absence of direct antibacterial activity (Nikolajeva et al. 1996), local application of AMPD to the ulcers shortened the time of neutrophil infiltration. It is possible that AMPD stimulated some or various cells of the local immune system in common with the systemic (i.v., i.m. or i.p.) administration of AMPD (Nikolajeva et al. 1996; 1999; 2000). However, we can not eliminate the possibility that AMPD has an impact also or solely on other cells of skin origin and the ulcer environment. Immunomodulation of the injury response may play a positive role not only with respect to the phase of inflammation, but also to the regeneration. Many of the growth factors present at a wound site can act as mitogens and/or as chemotactic factors for wound fibroblasts (Martin 1997). The involvement of the immune system in the response to tissue injury has raised the possibility that it might influence tissue, organ or appendage regeneration following injury (Godwin, Brockes 2006).

At present, our interests are focused on macrophages, as AMPD possesses stimulating effect on their activity (Nikolajeva et al. 1999). Also for example, chitosan, another well-known activator of macrophages, achieved recommendation for wound healing praxis (Mori et al. 2005). Moreover, most of the factors that regulate wound healing are glycoproteins (Gagneux, Varki 1999), and carbohydrates constitute about 20 - 25 % of the AMPD composition according to our previous study (Nikolajeva et al. 1996). Through interaction with lymphocytes, macrophages play an important role in the initiation and regulation of immune response. Activated macrophages release various substances such as cytokines and reactive intermediates and then carry out nonspecific immune responses (Son et al. 2001). In contrast, some studies suggest that "macrophageless" mice are able to repair skin wounds with a similar time course as wild-type siblings, and that repair appears scar-free as in the embryo, which also heals wounds without raising an inflammatory response (Martin et al. 2003). The scientific underpinnings for healing are better understood than ever, although much remains to be discovered. Eventually, an improved understanding of cellular and subcellular physiology may lead to new or better forms of therapy (Baum, Arpey 2005).

Hydrogels have been used primarily in the pharmaceutical field as carriers for delivery of various drugs, peptides and proteins therefore Carbopol gel was chosen as a carrier for AMPD also in our experiments. Hydrogels offer good opportunities due to their inherent biocompatibility. They maintain an appropriate moist environment, which is considered to accelerate wound healing. Their hydrophilic, soft and rubbery nature ensures minimal tissue irritation and a low tendency of cells and proteins to adhere to the hydrogel surface (Chu et al. 1992; Van Tomme, Hennink 2007).

Another relevant question involves the enzymatic activity of AMPD, i.e., deamination of adenosine and their derivatives. Adenosine is a well-known important signaling molecule that is released, for example, under inflammatory conditions. It can show anti-inflammatory as well as pro-inflammatory activities, and the contribution of the specific adenosine receptor subtypes in various cells, tissues and organs is complex (Akkari et al. 2006). Certainly, native AMPD binds to their substrates and deaminates. However, according to our previous studies, AMPD affects the immune response mainly without expression of their enzymatic activity (Nikolajeva et al. 1999). Our future experiments are directed to determine the mechanisms involved in the wound healing activity of AMPD.

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