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ENHANCING SKIN WOUND HEALING BY DIRECT INTRACELLULAR ATP DELIVERY

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Abstract

Background—A new intracellular adenosine triphosphate (ATP) delivery technique has been developed and was tested for skin wound care.

Methods—Eleven pairs of adult nude mice were used. ATP-vesicles were applied in 11 mice and another 11 mice were treated with lipid vesicles only.

Results—The group treated with ATP-encapsulated fusogenic small unilamellar lipid vesicles healed faster than the group treated with only lipid vesicles. Histologic study indicated a better developed granular tissue and reepithelialization in the study group, and wound tissue vascular endothelial growth factor (VEGF) expressions were also higher in this group.

Conclusions—This intracellular ATP delivery system may provide a new hope for wound healing as well as the treatment of medical conditions where ischemia is involved.

At least 3 million Americans suffer from chronic wounds, and many of these stem from diabetes, poor functioning veins, or pressure sores. The expense to the US health care system is an estimated \$7 billion dollars per year [1]. Of the 17 million Americans with diabetes [2], approximately 2.5–4.5 million (15–25%) will develop a chronic diabetic wound in their lifetime [3]. Despite thousands of remedies available to treat chronic wounds, none has proven effective [3].

One of the major pathophysiologic events in slow- or non-healing wounds is a deficient blood supply; the affected tissue exists in a partially ischemic state [4]. This decreased blood and oxygen delivery to the wound cells results in a significant decrease in cellular energy supply [5], which negatively impacts nearly every aspect of the healing process [6].

We have developed a new intracellular ATP delivery technique in which highly fusogenic lipid vesicles (ATP-vesicles) are used to encapsulate magnesium-adenosine triphosphate (Mg-ATP). The diameter of lipid vesicles is around 100–120 nm, and the phospholipids are similar to those in the cell membrane. When these lipid vesicles are in contact with the cell membrane, they fuse together and deliver their contents into the cytosol. The direct intracellular delivery

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of ATP may circumvent tissue ischemic damage. This article reports our results using the ATP-vesicles in full-thickness skin wound treatment in nude mice.

Methods

The study was approved by the Animal Care and Use Committee of the University of Louisville. A total of 28 nude mice (14 pairs) weighing 20–30 gm were used. Twenty four additional pairs of nude mice were used for measuring wound tissue VEGF. Under general anesthesia (sodium pentobarbital, 50 mg/kg, ip) and aseptic technique, a 6-mm diameter full-thickness skin wound on the head was made with a stainless steel punch. The wound was then covered with a piece of sterile cotton and Tegaderm™ (3M, Minneapolis, MN). The cotton was saturated with ATP-vesicles (4 mg/ml of DOPC/DOPC-E with 25 mM of Mg-ATP) or lipid vesicles only (control). The dressing was changed every day with either fresh ATP-vesicles or vesicles. The wound was documented with a digital camera. The wound area was calculated using NIH image software, digital images, and an internal scale measure.

To determine whether the effect of ATP-vesicles was by chance or by skin contraction only, one pair was sacrificed at day 12 for histological analysis, and another pair was switched after day 2. Another pair had incomplete observation and was also excluded. The remaining 11 pairs of nude mice were statistically analyzed.

Results

As shown in Figure 1, in all of the animals, ATP-vesicles significantly ($p=0.00029$) reduced the wound area and healed the wound quicker than in the control mice. The average healing time in the study group was 12.27 ± 0.48 days compared to 16.91 ± 0.87 days in the control group. Various digital images are depicted in Figures 2. In Figure 3, the wound in the study mouse was much smaller than the control animal after only one application of ATP-vesicles. We switched the animals so that the smaller wound became the control and the larger wound became the study. At days 2 and 3 after they were switched, the larger wound (now in the study mouse) became even larger. However, after day 8, the study wound gradually healed faster than the control wound.

One pair of mice was used for histological evaluation. Healing of the epithelial surface was more complete in the ATP-vesicles-treated mouse, with a thicker epidermal layer and no eschar. It had a thick layer of keratinized epithelium with no dimpling or distortion; there were more granular tissue formation in this wound sample and the granulation tissue was uniform and well organized. In the control mouse, there was a fragile, thin layer of poorly organized epithelium covered by eschar, and the partially healed wound had less granulation tissue which was also less well organized (Fig. 4). The clear and main difference was that wound treated with ATP-vesicles had much better granular tissue formation and epithelialized much faster and therefore much more completely by day 12, the day of harvest.

To examine the possible mechanisms by which ATP-vesicles enhanced wound healing, an additional 24 pairs of nude mice were used in which the wounds were treated the same way as above except that the wound tissue samples were removed at days 0, 1, 3, 5, and 7 (6 pairs each at each time point) for tissue VEGF expression study. The expression of VEGF was significantly higher in the wounds receiving ATP-vesicles than in those that received vesicles only after day 3 (Fig. 5). We also measured wound tissue ATP contents in these animals and the group treated with ATP-vesicles had higher ATP content in the wound tissue 3 days after the applications (data not shown).

Comments

The results of these preliminary experiments show that direct intracellular delivery of ATP by a high fusogenic carrier can accelerate the healing process in full-thickness skin wounds in nude mice. The most well-known factor that affects wound healing is available blood supply, which brings in oxygen, nutrients, minerals, enzymes, and circulating hormones [7]. The relative contribution and mechanisms for many factors affecting wound healing are not well studied [8]. However, one well-known mechanism is that all wound tissues are in a hypoxic state due to insufficient blood supply [9]. The wound architecture is partly controlled by the energy needs of the wound cells. An important factor in wound healing is collagen, which is synthesized by fibroblasts [9], which can survive but not replicate or synthesize collagen in low oxygen [10]. All other proteins needed for wound healing also require energy to synthesize [10]. The use of hyperbaric oxygen (HBO) is based on the principle that increased oxygen can help reduce infection and improve the tissue energy supply, but the true value of the increased atmosphere oxygen tension on local wound healing has yet to be established [11]. There are many reasons why, including the fact that oxygen alone is not enough for energy (mainly ATP) production [12], because other substrates (carbohydrates, lipids, and proteins) from blood circulation are equally important [13]. Cells use almost entirely ATP (in the form of Mg-ATP) for their survival and function [14]. In the wound area, decreased blood and oxygen delivery to the cells results in a significant decrease in cellular ATP [5]. To compensate for that, anaerobic glycolysis is increased in wound tissue [15]. However, this is inefficient for energy production, yielding only 1/16 the amount of ATP compared to aerobic energy production [16]. This decreased availability of ATP negatively impacts nearly every aspect of the healing process [6].

Our hypothesis is that, if we can deliver ATP into the cytosol, wound healing process will be greatly enhanced. In the literature, there have been other studies on the extracellular ATP application for wound healing. These reports have indicated that extracellular ATP and ADP are mitogenic factors that enhance DNA synthesis and have synergism with growth factors (eg, EGF, TGF- α) during wound healing [6;17]. However, there have been no studies to our knowledge in the literature that have focused on the effect of intracellular ATP on wounds.

All cells are surrounded by a plasma membrane, and the internal composition of the cell is maintained because the plasma membrane is selectively permeable only to small molecules. Specific transport proteins (carrier proteins and channel proteins) are needed for the selective passage of small molecules across the membrane. Larger, especially charged, molecules, for which no specific transport mechanisms exist, cannot cross cell membranes under normal conditions [18]. Unfortunately, ATP and many energy-rich glycolytic intermediates belong to this category.

To bypass the plasma membrane barrier, permeabilization [19], microinjection [20], and polymer delivery [21] have been developed for drug delivery, but none of these has yet achieved effective intracellular delivery of drugs. One promising approach is the loading of medication into liposomes, or multilamellar vesicles, which are microscopic sacs made of the very phospholipids that constitute cell membranes [22]. There are four types of interactions between liposomes and cell membrane [22]: adsorption, endocytosis, lipid exchange, and fusion. Since the discovery of liposomes in 1961 by Bangham et al [23] numerous reports have indicated the effectiveness of this technique in various treatment modalities, including anti-cancer therapy, anti-infection therapy, in vitro fertilization, diagnostic imaging, and gene therapy. For most of these results, the mechanism of delivery is related to adsorption, endocytosis, and lipid exchange. For membrane fusion, optimal conditions remain elusive and controversial. Three major problems have limited the widespread use of liposomes for cytosol drug delivery: 1) Liposomes are not readily fusogenic, mainly because the stored energy of the vesicles' radius

of curvature is minimal, and the internal layers may inhibit fusion [24]. Studies have shown that large unilamellar vesicles can only fuse with cells after electroporation, and multilamellar liposomes do not fuse with cells even after electroporation [24;25]. 2) Because of their size, most intravenously infused liposomes are unable to leave the general circulation, except in areas where vessels become leaky (such as hypoxia or inflammation) [26]. 3) The body's immune system recognizes the liposomes and removes them from circulation regardless of the vesicles' composition and size. The liver, spleen and bone marrow take up nearly all liposomes given intravenously, preventing liposomes from circulating long enough to reach targeted cells and tissues efficiently [26]. To circumvent these problems, various techniques have been developed, such as coating liposomes with polyethylene glycol [27], polyvinyl alcohol [28], or other polymers [29], to make them "stealth"; and combining liposomes with hemagglutinating virus of Japan (HVJ) to enhance fusion [30].

One important aspect of liposome research still lacking is the effort to alter the membrane bilayer properties and particle size to increase vesicle-cell fusion. There are many factors that affect the successful delivery and fusion of the lipid vesicles, including cell-cell adhesions, extracellular matrix, non-specific binding, phagocytic cells, and the availability of water and ions. In certain applications, such as in topical wounds, many of these factors are less important. In solid organs, such as the heart and liver, these factors play a key role in the successful delivery of ATP. Furthermore, the fusion of the lipid vesicles can have adverse effects on the target cell. We have found that there are a variety of means available to alter the fusion rate of the ATP-vesicles to maximize delivery of ATP and minimize any adverse effects of the fusion event on the cells. We have also had success optimizing the size of the vesicles in order to achieve a balance between the amount of ATP encapsulated inside of the vesicle and the fusogenic nature of the vesicle. Our newest vesicle preparations have an average diameter of only 70 nm, much smaller than the liposomes reported in the past. By encapsulating magnesium-ATP inside of highly fusogenic lipid vesicles, we can deliver ATP at rates sufficient to match the ATP demand of the target cells. In our cell culture studies, these lipid vesicles were shown to deliver encapsulated carboxyfluorescein into the cultured endothelial cells within less than 5 minutes (data not shown).

A direct intracellular delivery of Mg-ATP into wound cells can bypass the need for a fully intact blood circulation and still provide the much-needed energy to all the starved cells to facilitate wound healing. We are not sure which cell or cells will benefit most. However, the application of ATP-vesicles resulted in significant wound area reduction, especially within the first 1 to 5 days of application. This period correlates with skin contraction, macrophage stimulation, fibroblast proliferation, and other fibroblast-dependant activities such as collagen synthesis and extracellular matrix production. Wound healing involves a complex interaction between epidermal and dermal cells, the extracellular matrix, controlled angiogenesis, and plasma derived proteins; and successful healing depends on skin contraction, granular tissue formation, and re-epithelialization [31]. ATP-vesicles appear to benefit all these factors during the healing process. As seen from our results, the early fast reduction of wound size was most likely the result of skin contraction, but the rich granular tissue production and successful re-epithelialization in the ATP-vesicles treated wound at day 12 indicated the contribution of these two factors. The finding that the highly efficient granular tissue production in the study group should also be very attractive in clinical practice because, in many surgical cases, tissue defects need to be filled by granular tissue before re-epithelialization is completed.

The approach reported here has many advantages: it is easy to apply, it has minimal expense, and it has low toxicity. The use of small unilamellar vesicles is not our innovation, nor is the encapsulation of ATP into liposomes. Our innovations are: (1) the use of small unilamellar vesicles that are highly fusogenic to encapsulate ATP and allow efficient cytosolic delivery of ATP, and (2) to use intracellular energy delivery for wound healing. To our knowledge, this

is the first report of using fusogenic small lipid vesicles to deliver ATP directly into wound cells. If this approach is proven successful in acute as well as chronic wounds, it will benefit millions of patients with diabetic wounds, pressure ulcers, and other types of acute and chronic wounds.

There are limitations to this study. First, the validity of the nude mouse model has been questioned because nude mice do not have complete thymus function and are therefore viewed as immuno-deficient animals. We selected this species because they were easy to handle, and it was simple to attach dressings to their hairless skin. They have a considerably higher macrophage content that may compensate for their T-cell deficiency. In our study, no single infection had occurred in any mouse. As such, we did not perform bacterial culture study. Our experience is very much in line with many previous wound studies in which nude mice were successfully used for various wound studies [32–34]. Second, this model mimics the acute wound healing process in healthy animals. We are not sure if this result can be extrapolated into chronic wounds because the latter normally have underlying vascular or neural diseases that are the basis for many skin ulcers such as diabetic, ischemic, or pressure ulcers. Theoretically, the low-oxygen and low-energy circumstances are usually worse in chronic wounds, such as in diabetic wounds, in which bioenergetic metabolites are lower in diabetic patients than in normal controls [35], a direct intracellular ATP delivery should benefit these wounds as well. However, more experiments using chronic wound models are definitely needed to evaluate the usage of this technique.

Although this preliminary result is promising, we consider this to be the early stage of our study. Numerous previous reports showed very promising results using some chemicals in animals but latter more extensive animal experiments or clinical trials did not substantiate the early findings. As stated earlier, wound healing is a complex process and many factors contribute to the successful closure of a wound, we should be very cautious in claiming the effectiveness of any new technique. Besides, the mechanisms of wound healing are quite different between humans and rodents [36], and larger animal experiments are needed to further substantiate the results. On the other hand, in view of the fact that there is no effective technique to enhance wound healing, the results obtained from our study are encouraging. The increased wound tissue VEGF expressions in the mice treated with ATP-vesicles indicated that, with a direct intracellular ATP delivery, it is possible that a healthier environment is provided for accelerated healing.

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Figure 1.
Comparison of wound healing time between the study group versus controls.

Figure 2.
Examples of wound healing in 6 pairs of nude mice.

Figure 3.

In one pair of nude mice, the animals were switched after day 1. The study animal still healed quicker than the control animal.

Figure 4. Histological comparison of one pair of nude mice at day 12 after treatment with ATP-vesicles (top) compared with control mouse (bottom).

Figure 5.
Comparison of wound VEGF expression between the two groups.