Biochemical and Biophysical Research Communications 500 (2018) 665–670

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Molecular and histological study on the effects of non-thermal irreversible electroporation on the liver

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ARTICLE INFO

Article history:
Received 3 April 2018
Accepted 17 April 2018

Keywords:
Non thermal irreversible electroporation
Liver
Cell death mechanism
Minimally invasive regenerative surgery

ABSTRACT

Non-thermal irreversible electroporation (NTIRE) is a biophysical phenomenon in which certain electric fields delivered across the cell membrane in tissue, cause cell death, without affecting the extracellular matrix. “Minimally invasive regenerative surgery” is a new medical modality for treatment of end-stage organ or tissue failure in which exogenous cells are implanted in a decellularized niche in tissue, formed by the delivery of NTIRE electric fields across a targeted volume of tissue. We anticipate that the success of the procedure will depend on the time of implantation relative to the application of NTIRE. This study was performed to elucidate the histological and molecular events that occur within 24 h after NTIRE, in the context of optimal criteria for the time of implantation. To this end, we examined the histology of NTIRE treated rat liver with H&E, Masson trichrome and TUNEL staining. Western blot was used to examine pro and cleaved caspase-3 (marker for apoptosis), pro and cleaved caspase-1 and gasdermin D (markers for pyroptosis), and RIP3 and MLKL (markers for necroptosis). The key findings are that, complete hepatocytes disintegration within an intact extracellular matrix is seen at 6 h and, new hepatocytes are seen in the treated region at 24 h, after NTIRE. There is no evidence of apoptotic cell death from NTIRE, contrary to commonly made claims in the NTIRE literature. However, molecular pathways of pyroptosis and necroptosis, programed necrosis associated with inflammation, are activated at 6 h after NTIRE and are not evident at 24 h after NTIRE. These are fundamental new findings of basic value to the field of NTIRE in all its applications. Taken together the results suggest the hypothesis that an optimal time for implantation is about 24 h after NTIRE. Future studies in which exogenous cells are implanted at different times after NTIRE are required to examine this hypothesis.

1. Introduction

Recently [1], we have pioneered “minimally invasive regenerative surgery” (MIRS) as a new medical modality that could become an alternative to whole organ transplant for treatment of end-stage organ failures, such as the liver, or for treatment of impaired organ function, such as diabetes. MIRS employs non-thermal irreversible electroporation (NTIRE), to create an *in vivo* niche of a decellularized intact extracellular matrix for exogenous cell engraftment [1]. Electroporation, is the permeabilization of the cell membrane with electric fields, delivered across a cell [2]. Electroporation can be reversible [3], in which case the cell returns to its original state a certain time after the electric field has ceased, or irreversible [4], when the cell succumbs to the permeabilization of the cell membrane. Non thermal irreversible electroporation (NTIRE), has recently acquired importance in minimally invasive ablation surgery of otherwise unresectable tumors, e.g. Refs. [5,6] on the strength of its ability to cause cell death by selectively affecting only the cell membrane, while sparing the extracellular matrix and important tissue features [7]. Notably, this has led to the

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https://doi.org/10.1016/j.bbrc.2018.04.132
0006-291X/

idea that treating tissues with NTIRE could be used to form a decellularized tissue scaffold, that serves as an *in vivo* generated niche for exogenous cell engraftment [1].

The optimal time for exogenous cell engraftment after NTIRE, is a key parameter in MIRS. In the liver, the optimal time for implantation should satisfy the following criteria: 1) the hepatocytes have disintegrated, to avoid encroaching the newly transplanted cells; 2) the extracellular matrix maintains a comparatively normal architecture to support the implanted cells; 3) inflammatory response are mild, so as to minimize short term immune response damage to the implanted cells. However, currently, the majority of studies on NTIRE focus on the long term effects, because these are important to clinical applications in the treatment of cancer. There is very little research on the processes which occur at the early stages after the NTIRE treatment, which is the time of relevance to MIRS. The goal of the study reported in this paper was to generate fundamental information on the physiological and molecular events which occur within the first 24 h after NTIRE, to provide support for future studies in which exogenous cells will be implanted in the NTIRE formed niche at the optimal time suggested by this study. We have used H&E and Masson's trichrome stains to follow the physiological events that occur after NTIRE and to determine the optimal time for criteria 1) and 2) above. Concerning molecular events, the majority of publications on NTIRE, credit cell death to apoptosis, primarily on the basis of TUNEL stain based experiments e.g. Refs. [5,8]. In regards to criteria 3) avoidance of inflammation, it is important to confirm that cell death is by apoptosis or not. Apoptosis generates a complex mixture of cell surface–associated molecules that are released by or displayed on apoptotic cells to help phagocytes find and engulf them without triggering inflammation [9]. In contrast cells undergoing necrotic death rupture release factors that stimulate inflammation. Pyroptosis and necroptosis are two inflammatory programed necrosis molecular pathways that involve membrane rupture and release of cytoplasmic contents and are distinct from apoptosis. Pyroptosis is driven by the inflammatory caspases: caspase 1, 4, 5 and 11. Once caspases 1, 11, 4 or 5 have been activated, they trigger pyroptosis by cleaving gasdermin D [10]. Activation of caspase 1 and failure to activate caspase 3 (which is associated with apoptosis) are considered a key marker of pyroptosis [11]. Necroptosis is also a programmed form of necrosis that is dependent on activation of receptor-interacting kinase (RIPK3) [12] and the mixed lineage kinase domain-like (MLKL) [13]. In this study, we examined molecular markers for apoptosis, pyroptosis and necroptosis, throughout the first 24 h after NTIRE, in the context of 3) above.

2. Materials and methods

Sprague–Dawley rats weighing 250–350 g were used in this study. All animals received humane care from properly trained professionals in compliance with both the Principals of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, published by the National Institute of Health (NIH publication no. 85-23, revised 1985), and treated according to an animal protocol approved by the Animal Care and Use Committee of the University of California, Berkeley.

The surgical procedure followed a protocol described in detail in Ref. [14]. The animals were anesthetized, and the peritoneal cavity is entered via a midline incision of the abdomen. Three lobes were treated in each animal and each lobe was treated once. After partial mobilization of the liver from adjacent tissue, the treated liver lobe was gently clamped between the two 10 mm diameter electrodes, separated by 3 mm (Harvard Apparatus, Holliston, MA, USA), as described in Ref. [14]. A sequence of 10 square pulse with an electric field of 1000 V/cm, 100 μs pulse width, separated by 100 ms was applied between the electrodes, across the liver, using an electroporator (ECM 830, Harvard Apparatus, Holliston, MA, USA). (Previous studies with the experimental configuration in this study have shown that this electroporation protocol produces minimal thermal damage, and the tissue is affected by irreversible electroporation only [15].) At the end of the NTIRE experiment, the abdomen wall was closed and sutured. We examine the liver via liver harvest at the following times after the procedure: 1 h, 3 h, 6 h, 24 h.

For histological examination, tissue samples were cut normal to the liver lobe surface, distal to the treated region and through the center of the treated lesion, fixed in formalin, embedded in paraffin and sectioned 5- μm thick. They were stained with hematoxylin and eosin, Masson's trichrome, and TUNEL assays by (Histo-Tec Laboratory, Hayward, CA, USA) and (Histowiz Inc, Brooklyn, NY, USA).

The liver tissues were lysed, and the lysates were subjected to Western blot assays. Anti-caspase-1 and anti-RPI3 were purchased from Abcam, anti-GSDMD was purchased from abbexa, anti-MLKL was purchased from Abclonal, anti-caspase-3 was purchased from Cell signaling technology; and visualized using the Western Bright ECL detection system (Biorad Hercules, CA) after incubation.

3. Results

We have performed four repeats for each experimental condition and the results shown here are typical to all the repeats. Continuous monitoring of the animals indicated that the animals did not experience any adverse effects due to the non-electrolytic NTIRE treatment procedure.

Fig. 1 shows the histology of the H&E stained liver in different regions of the treated liver at different times after NTIRE treatment, as listed in the figure legend. Panel 1-A1 shows the normal liver and arrows point to a normal hepatocyte, an endothelial cell and a Kupffer or macrophage cell. Panel 1-A2 shows that hepatocytes in the treated and untreated part of the liver appear normal and there is no distinct interface between the treated and untreated areas. However, there seem to be more Kupffer cells or macrophages in the area corresponding to the treated tissues. Panels 1-A3 and 1-A4 show a relative large number of Kuppfer cells or macrophages, expanded sinusoids, and some ballooned but intact hepatocytes. Panels 1-B show micrographs taken 3 h after the treatment. The H&E staining shows more ballooned hepatocyte than after 1 h, vascular congestion and hemorrhagic change in ablation area. Cytoplasmic limits between the cells are barely distinguishable. Kupffer cells infiltration is seen. There are sites at which only the extracellular matrix is seen with no cell structure. It is interesting to notice in panel 1-B3 that the large blood vessels, (portal vein, hepatic artery and bile duct structure appear morphologically intact). Panels 1-C show the H&E stained tissue 6 h after NTIRE. Panel 1-C2 shows a clear, cell scale resolution, line of demarcation between the non-electrolytic NTIRE treated zone (right hand side) and the normal liver (left hand side). The difference in appearance between the treated and untreated regions is striking. In the normal liver the sinusoids are patent and the hepatocytes look normal. In the treated region, which is separated from the normal liver by only several layers of cells, the liver architecture is completely lost and vesicle without nuclei and inflammatory cells are seen throughout. The treated region has experienced severe congestion and hemorrhage. Panels 1-D show the H&E stained region 24 h after NTIRE. Panel 1-D2 shows that the distinction between the treated and untreated has become vague, strikingly different from the pronounced margin 6 h after the treatment. In the untreated zone, the tissue structure is comparatively normal, with some nonspecific cell swelling and inflammatory cell infiltration. In the ablation zone,