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Immunohistochemical Identification of Alpha-Smooth Muscle Actin Positive Myofibroblasts in Toxic Hepatitis

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Abstract. The crucial role played by the myofibroblast in wound healing and pathological organ remodeling is well established. Hepatic myofibroblasts constitute a heterogeneous population of pro-fibrogenic cells found in chronically injured livers. Liver fibrogenesis is sustained by populations of highly proliferative, pro-fibrogenic, pro-angiogenic and contractile myofibroblasts that, originate by a process of activation involving perisinusoidal hepatic stellate cells, portal fibroblasts, bone marrow-derived mesenchimal stem cells as well as, in certain conditions, by a process of epithelial to mesenchymal transition involving hepatocytes and cholangiocytes. (Parola 2008). In this study we used 60 male Wistar rats with an average weight of 200g, who received chronic carbon tetrachloride treatment in dose of 1,2 ml/kg, two times a week, for 12 weeks. Liver samples were taken after euthanasia at 4, 8 and 12 weeks after the start of the experiment. The formalin-fixed hepatic samples were embedded in paraffin, sectioned at 4 µm, stained by Haematoxilin-Eosine and Masson's trichrome methods. Immunohistochemistry was performed, using a Rabbit polyclonal antibody to alpha smooth muscle Actin (Abcam, Cambridge, ab5694). Alpha smooth muscle actin (SMA) positive myofibroblast were localized in the expanded connective tissue around central veins, within fibrous septa, at the interface between fibrous septa and parenchyma and in the parenchyma around sinusoids (activated hepatic stellate cells).

Keywords: myofibroblast, fibrogenesis, rat, immunohistochemistry, alpha smooth muscle actin.

INTRODUCTION

Wound healing is characterized by the presence of a cell called myofibroblast that is responsible for pathological tissue remodeling. The myofibroblast has been initially identified by means of electron microscopy in granulation tissue of healing wounds as a modulated fibroblast exhibiting features of smooth muscle cells.(Gabbiani, 2004).

Fibrogenesis is initiated by hepatocyte damage leading to a recruitment of inflammatory blood cells and platelets as well as activation of Kupffer cells with subsequent release of different cytokines. Hepatic stellate cells (HSC) seem to be the primary target cells for inflammatory stimuli (Knittel, 1999).

Liver fibrogenesis is sustained by a heterogeneous population of highly proliferative, pro-fibrogenic, pro-angiogenic and contractile myofibroblasts that, originate by a process of activation involving perisinusoidal hepatic stellate cells, portal fibroblasts, bone marrow-derived mesenchimal stem cells as well as, in certain conditions, by a process of epithelial to mesenchymal transition involving hepatocytes and cholangiocytes (Parola, 2008, Parola

2009). This cells are found in chronically injured livers. The main mediator of this myofibroblastic activation is represented by TGF β (transforming growth factor β) (Tache, 2010). Alpha-smooth muscle actin, the actin isoform typical of vascular smooth muscle cells, is the main marker of the myofibroblastic differentiation.(Gabbiani, 2004).

MATERIALS AND METHODS

In this study, we used 60 male Wistar rats (Rattus norvegicus, strain Wistar) of an average weight of 200g, divided in two groups.

In the **first** group 45 rats were distributed. At this group we performed a chronic carbon tetrachloride (CCl_4, conc. 25%) administration given by oral gavage, in a dose of 1,2 ml/kg, twice a week.

In the **second** group, the control one, 15 animals were distributed, they received saline solution given by oral gavage in a dose of 1,2 ml/kg, two times a week.

The animals were kept at room temperature, with 12 hours light-dark cycle, food and water *ad libitum.*

Liver samples were taken after euthanasia at 4, 8, and 12 weeks after the start of the experiment. After 4 weeks 15 animals from the first group and 5 from the control one were sacrificed, at 8 and 12 weeks the same number of animals were sacrificed from the two groups.

Liver samples were fixed in 10% neutral buffered formalin and routinely embedded in paraffin, the sections made at 4 micrometers with a microtome Leica RM 2125 RT and stained by Haematoxiline–Eosine and Masson's trichrome methods.

Immunohistochemistry was performed using a Rabbit polyclonal antibody to alpha smooth muscle Actin (Abcam, Cambridge, ab5694). For immunohistochemistry sections (4 μm) were mounted on poly-L lysine coated slides and stored for a maximum of 48 hours at room temperature until use. After that slides were deparaffinized in xylen. Antigen retriever had been realized using a pressurized cooker in citrate solution, pH=6.0 (Dako); endogenous peroxidase was inactivated by peroxidase blocking reagent (Dako - Peroxidase and PA blocking reagent 3%) during 5 minutes at room temperature. Primary polyclonal antibodies (anti-α SMA) were maintained overnight, during 18 hours at 4°C, using a dilution of 1:200 (Dako antibody diluent). The visualization of immunological reaction was performed using Universal LSAB+Kit/HRP, Rb/Mo/Goat (DAB+) system (Dako); the counterstaining was performed using Mayer's hematoxylin. To evaluate the antibody specificity were used negative control (replacing the primary antibody with antibody diluent) and internal positive tissue control.

The slides were examined under a microscope Olympus BX 51,the images were taken with Olympus DP 25 digital camera and processed by a special image acquisition and processing program: Olympus Cell B.

RESULTS AND DISCUSSION

Slides stained by Haematoxiline-Eosine and Masson's trichrome methods were examined for assessing histological changes. Distribution of α-smooth muscle actin positive myofibroblast was assessed immunohistochemically.

At 4 weeks central fibrosis with formation of central-central fibrous bridges was present (Fig.2.). Hepatocellular necrosis and ballooning degeneration of hepatocytes, especially around central veins, were evident (Fig.1.). It is also present a polimorf

inflammatory cell infiltrate dominated by neutrophils, indicating an active chronic inflammatory response to persistent hepatocyte necrosis. Numerous macrophages with ceroid were present in the fibrous central expansions and within fibrous septa. Bridging fibrosis with active fibrous septa formed between adjacent terminal hepatic venules delimitate nodules. Scattered foci of intralobular necrosis with the presence of lymphocytes were noticed.

At 8 weeks after the start of the experiment, the lesions were dominated by central-central bridging fibrosis (Fig.4). The central-central bridges were represented by active fibrous septa, with abundent inflammatory infiltrate especially with neutrophils and mononuclear cells. In the central fibrous expansions and within fibrous septa, numerous macrophages loaded with ceroid were present (Fig.3).

After 12 weeks, at histological examination the dominant feature was represented by central-central bridging fibrosis, the necro-inflammatory activity being less evident. Within fibrous septa ceroid loaded macrophages were present.

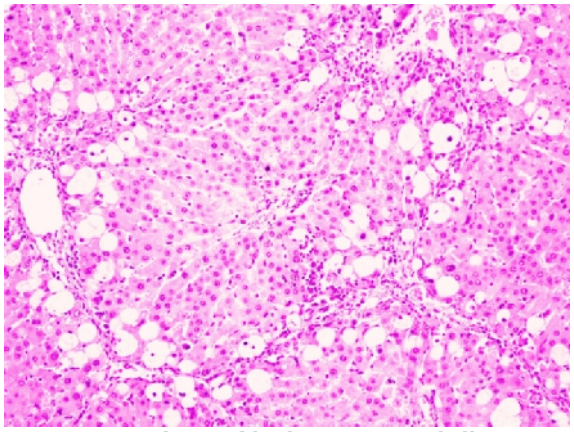

Fig.1. Central-central bridging necrosis, balloning degeneration of hepatocytes, (Experimental group, 4 weeks of CCl_4 administration), HE x100.

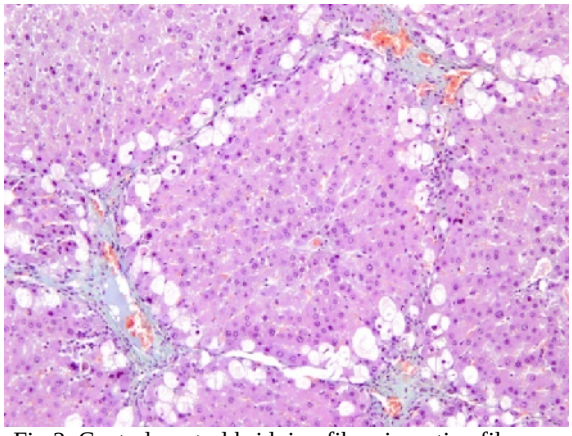

Fig.2. Central-central bridging fibrosis, active fibrous septa, ballooning degeneration of perivascular and periseptal hepatocytes, macrophages loaded with ceroid within fibrous expansions (Experimental group, 4 weeks of CCl_4 administration), Masson's trichrome, x100.

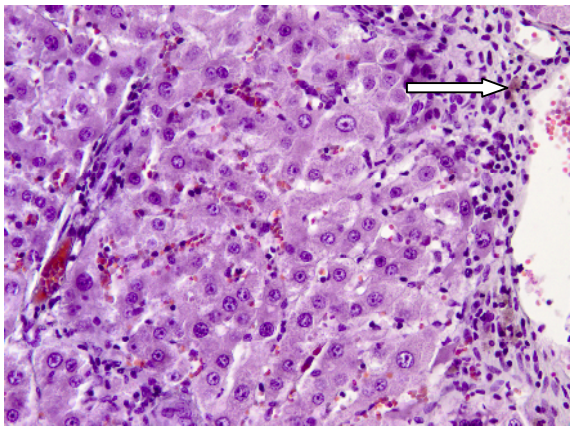

Fig.3. Central fibrosis, macrophages with ceroid in the central fibrous connective tissue mass (arrow), (Experimental group, 8 weeks of CCl_4 administration), Masson's trichrome, x400.

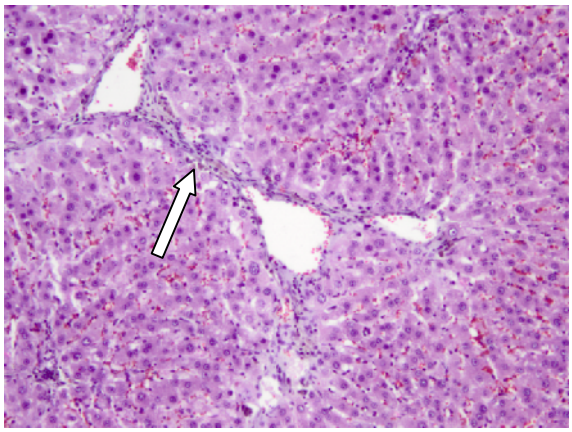

Fig.4. Central-central bridging fibrosis with active fibrous septa formed between adjacent terminal hepatic venules (arrow), (Experimental group, 8 weeks of CCl_4 administration), Masson's trichrome, x100.

Regarding the identification of α-SMA positive myofibroblast, we haven't noticed a significant difference between the hepatic samples collected at different intervals, respective at 4, 8 and 12 weeks after the beginning of the experiment. The distribution and number of the myofibroblasts were similar. Some difficulties occurred at the identification of positive immunoreactions due to the presence of ceroid loaded macrophages within fibrous septa and perivenular fibrous expansions which had a similar aspect with that of α-SMA positive cells. The differentiation of this two cell types was realized using fluorescent microscopy (the ceroid is auto fluorescent) and by the assessment of cell's morphology.

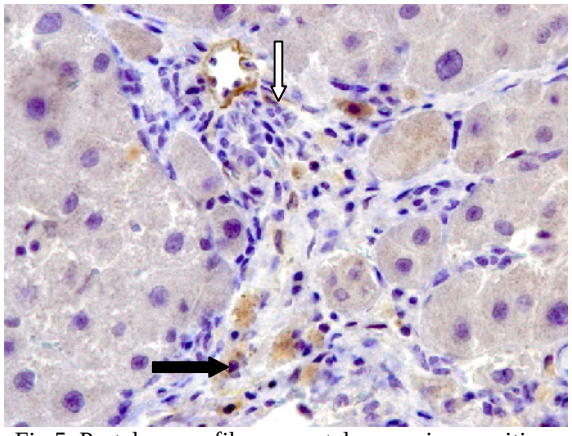

Fig.5. Portal space, fibrous portal expansion, positive immunolabelling of smooth muscle cells of vascular media, α-SMA positive myofibroblasts (arrow), ceroid loaded macrophages (black arrow), IHC reaction anti-α-SMA, counterstaining with Mayer's hematoxylin x400. (Experimental group, 12 weeks of CCl_4 administration)

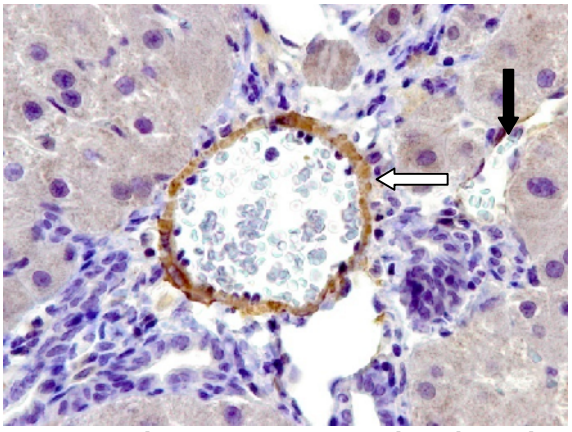

Fig.6. Portal space, positive immunohistochemical labeling of smooth muscle cells of vascular media (arrow), positive labeling of some endothelial cells (black arrow), IHC reaction anti-α-SMA, counterstaining with Mayer's hematoxylin x400. (Experimental group, 12 weeks of CCl_4 administration)

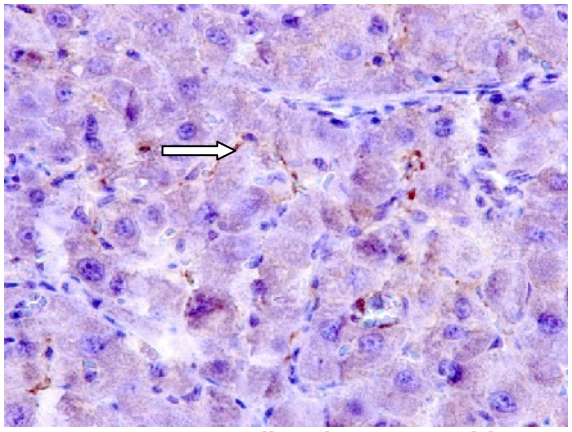

Fig.7. α-SMA positive cells in the perisinusoidal space (arrow), IHC reaction anti-α-SMA, counterstaining with Mayer's hematoxylin x400. (Experimental group, 8 weeks of CCl_4 administration)

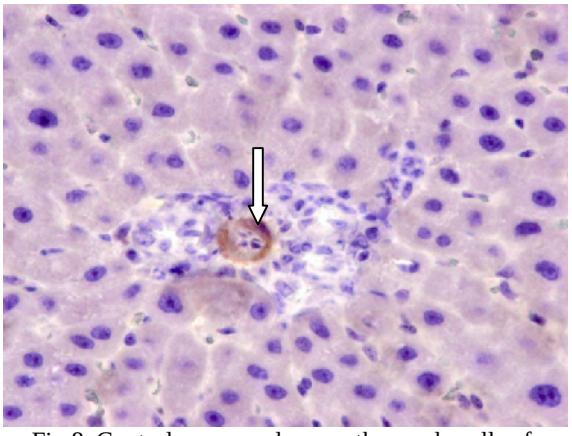

Fig.8. Control group, only smooth muscle cells of vascular media showing positive immunoreaction (arrow), IHC reaction anti-α-SMA, counterstaining with Mayer's hematoxylin x400. (Control group, 12 weeks of saline solution administration)

Three categories of α-SMA positive myofibroblasts were described: a) myofibroblasts localized in the central fibrous connective tissue, around portal spaces or within fibrous septa;

b) interface myofibroblasts localized at the interface of fibrous septa with parenchyma and c) perisinusoidal located α-SMA positive cells (probably activated hepatic stellate cells). Smooth muscle cells of the media of larger vessels, as well as some endothelial cells showed positive immune reaction.

No lesions were found during the examining of the control group, and at the immune histochemistry we noticed only a number of smooth muscle cells from the vascular media which showed positive immunoreactions.

CONCLUSSIONS

By using the immunohistochemical technique with antibodies against α-SMA the visualization of activated myofibroblasts was made possible. The majority of the myofibrolasts were located within the fibrous septa and the perivenular connective tissue mass.

The perisinusoidal located hepatic stellate cells showed inconstant immune positivity.

The ceroid loaded macrophages within the fibrous septa had to be differentiated from α-SMA positive cells, this two cell types presenting a resembling aspect.

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