ARTÍCULO ORIGINAL

Expression of TWEAK/Fn14 axis in the context of metabolic dysfunction associated-fatty liver disease: an approach in liver regeneration

Expresión del eje TWEAK/Fn14 en el contexto de la enfermedad del hígado graso asociada a disfunción metabólica: un enfoque en la regeneración hepática

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In addition to being the corresponding author, ASC had proposed the idea for research and had conducted a final revision of the gathered data. JBST, DRS and LLA and had collected the relevant data and had prepared the manuscript. MCG and ASC had outlined the study design and revised the manuscript. The authors read and approved the final manuscript.

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ABSTRACT

Background: One of the pathways involved in liver regeneration processes is TWEAK/Fn14 (tumor necrosis factor-related weak inducer of apoptosis/fibroblast growth factor-inducible 14), which has been proposed to act directly and selectively on hepatic progenitor cells; however, its role in the regeneration of steatotic liver metabolic dysfunction associated fatty liver disease has not been fully elucidated. **Objective:** To evaluate the behavior of Fn14 and its ligand TWEAK, as well as cellular stress signals as biochemical cues for possible liver regeneration in MAFLD. **Materials and methods:** A prospective study was carried out where the behavior of Fn14 and its ligand TWEAK, as well as cellular stress signals were observed as biochemical indications of a possible liver regeneration in a condition of tissue damage caused by excessive lipid accumulation. The expression of TWEAK, Fn14 and heat shock proteins in hepatic steatosis of non-alcoholic origin was assessed using immunohistochemistry and western blotting. **Results:** The histological classification of the tissues under study corresponded to microvesicular steatosis. We report a high level of expression of heat shock proteins in the cytoplasm. The expression of TWEAK and Fn14 in liver tissue affected by lipid accumulation was localized in the cytoplasm of hepatocytes, showing a higher intensity of reactivity for Fn14 compared to its ligand TWEAK. **Conclusion:** The expression of TWEAK/Fn14 axis was positive suggesting reactivity of the signaling pathway in metabolic dysfunction associated fatty liver disease.

Keywords: *Fatty liver; TWEAK receptor; Fn14 receptor; Liver regeneration (source: MeSH NLM).*

RESUMEN

Antecedentes: Una de las vías implicadas en los procesos de regeneración hepática es el eje TWEAK/Fn14 (inductor débil de la apoptosis similar al factor de necrosis tumoral/factor de crecimiento de fibroblastos inducible 14), el cual se ha propuesto que actúa directa y selectivamente sobre las células progenitoras hepáticas; sin embargo, su papel en la regeneración del hígado esteatósico asociado a la enfermedad metabólica del hígado graso no ha sido completamente dilucidado. **Objetivo:** Evaluar el comportamiento de Fn14 y su ligando TWEAK, así como señales de estrés celular como indicadores bioquímicos de posible regeneración hepática en MAFLD. **Materiales y métodos:** Se llevó a cabo un estudio prospectivo donde se observó el comportamiento de Fn14 y su ligando TWEAK, así como señales de estrés celular como indicaciones bioquímicas de una posible regeneración hepática en una condición de daño tisular causado por acumulación excesiva de lípidos. La expresión de TWEAK, Fn14 y proteínas de choque térmico en esteatosis hepática de origen no alcohólico se evaluó mediante inmunohistoquímica y western blot. **Resultados:** La clasificación histológica de los tejidos estudiados correspondió a esteatosis microvesicular. Reportamos un alto nivel de expresión de proteínas de choque térmico en el citoplasma. La expresión de TWEAK y Fn14 en el tejido hepático afectado por acumulación de lípidos se localizó en el citoplasma de los hepatocitos, mostrando una mayor intensidad de reactividad para Fn14 en comparación con su ligando TWEAK. **Conclusión:** La expresión del eje TWEAK/Fn14 fue positiva, sugiriendo reactividad de la vía de señalización en la enfermedad metabólica del hígado graso asociada a disfunción metabólica.

Palabras clave: *Hígado graso; Receptor de TWEAK; Receptor de Fn14; Regeneración (fuente: DeCS Bireme).*

INTRODUCTION

Fibroblast growth factor-inducible 14 (Fn14), is a receptor belonging to the tumor necrosis factor (TNF) superfamily for the tumor necrosis factor-related weak inducer of apoptosis (TWEAK) [1]. TWEAK is expressed by macrophages [2] and acts as a mitogen via Fn14 for liver progenitor cells [3-5]. Through TWEAK/Fn14 interaction, it promotes expansion of progenitor cells involved in liver regeneration [6].

In addition, activation of the TWEAK/Fn14 pathway stimulates pro-inflammatory responses in many diseases [7]. Murine studies suggest that the main function of Fn14 is to initiate ductal proliferation and expansion of hepatic progenitor cells through activation of NFK-β [6]. Fn14 is scarcely detectable in healthy adult liver, but induction of Fn14 expression has been observed in many types of liver damage, e.g., after partial hepatectomy [6], chronic liver disease, metabolic dysfunction associated fatty liver disease (MAFLD [formerly named nonalcoholic fatty liver disease; NAFLD]), steatohepatitis [8], and hepatocellular carcinoma [9]. Previous studies have shown that Fn14 is significantly more expressed in patients with fatty liver than other liver diseases and suggest a therapeutic target in this condition [10]. Histopathologically, MAFLD is characterized by excessive storage of macrovesicular fat in hepatocytes, deposits composed of triglycerides that in some individuals trigger an inflammatory response responsible for steatohepatitis, characterized histologically in turn by the presence of more than 5% of macrovesicular steatosis, ballooning of hepatocytes and presence of inflammation with predominantly centro-lobular distribution [11].

Although activation of the TWEAK/Fn14 pathway plays a critical role in the pathogenesis of steatosis, the mechanisms underlying this disease are poorly understood and much of what is known is from animal and cellular models [12]. Then, the aim of this study was to evaluate the behavior of Fn14 and its ligand TWEAK, as well as cellular stress signals as biochemical cues for possible liver regeneration in MAFLD.

MATERIALS AND METHODS

A prospective study was conducted that included samples from 8 men and 20 women with an age range between 25 and 73 years and an average age of 42 years. Clinical samples of liver tissue in steatosis condition were provided by the Pathology department of the participating institutions from June to November 2022; with pathological confirmation of MAFLD. Patients who presented excessive alcohol consumption (20 g/day for men and 10 g/day for women) were excluded, as well as women who were pregnant or taking oral contraceptives and corticosteroids at the time of the study. Likewise, patients with viral hepatitis, hemochromatosis, and Wilson's disease who were under treatment with hepatotoxic drugs were not considered.

Part of the tissue samples were fixed with 10% buffered formaldehyde for subsequent immersion in paraffin and staining with hematoxylin and eosin, the rest were stored at -80°C for subsequent analysis. Informed consent was obtained from all participants. The study was approved by the ethical committee of Universidad de Cartagena and conducted according to the declaration of Helsinki.

Immunohistochemical dyeing

Tissues were fixed in 10% formaldehyde and were embedded in paraffin, subsequently cut into 5-µm thick sections, carefully washed 0.01M PBS (Buffered Phosphate Saline) three times. To block endogenous peroxidases, the paraffin sections were treated with 3% hydrogen peroxide for 20 minutes. The slices were blocked with 2% Milk in 0.01M PBS with 0.3% triton X-100 at 1h at room temperature, then incubated at 4°C overnight antibody (rabbit o) against HSP70 (HSP 70, 1:100, mouse monoclonal clone 5A5, ab2787, Abcam® Inc, Cambridge, MA, USA), HSP90 (HSP90 1:100 monoclonal Sigma Aldrich), Fn14 (Anti-FN14 SAB5500106-100UL, Rabbit monoclonal recombinant, SIGMA-ALDRICH), TWEAK (Rabbit polyclonal antibody CUSABIO CSB-PA152148, dilution 1:100. The sections were then subjected to immunohistochemical dyeing with an enzyme-linked immunosorbent antigen detection system according to the manufacturer's instructions. Finally, the sections were developed with diaminobenzidine and observed under a Leica DM500 light microscope. The dyeing was considered positive when there was a strong brown dyeing in the epithelial membrane of the cells.

Western blot analysis

Protein extracts were prepared by homogenization of liver tissue in RIPA buffer and quantified by Bradford. Protein separation was performed by electrophoresis in polyacrylamide gels under denaturing conditions (SDS-PAGE). For immunodetection, anti-Fn14 primary antibody (1:1000 of Anti-FN14 antibody, Rabbit monoclonal recombinant, SIGMA-ALDRICH), and beta actin (Santa Cruz Biotechnology) were used, following the recommendations of the commercial company. Immunodetection was performed by chemiluminescence using horseradish peroxidase substrate in the IbrightCL1000 kit from Thermo scientific. For the descriptive analysis of the data, measures of central tendency, dispersion, and proportions were used. The IBM® SPSS® v.25 statistical package was used.

Ethical considerations

The ethical committee of Universidad de Cartagena approved and registered the research, and all patients signed an informed consent.

RESULTS

It was evaluated the behavior of Fn14 and its binding TWEAK, as well as cellular stress signals as biochemical indications of possible liver regeneration in a condition of tissue damage caused by excessive lipid accumulation in 28 steatotic liver tissues. To determine the protein expression

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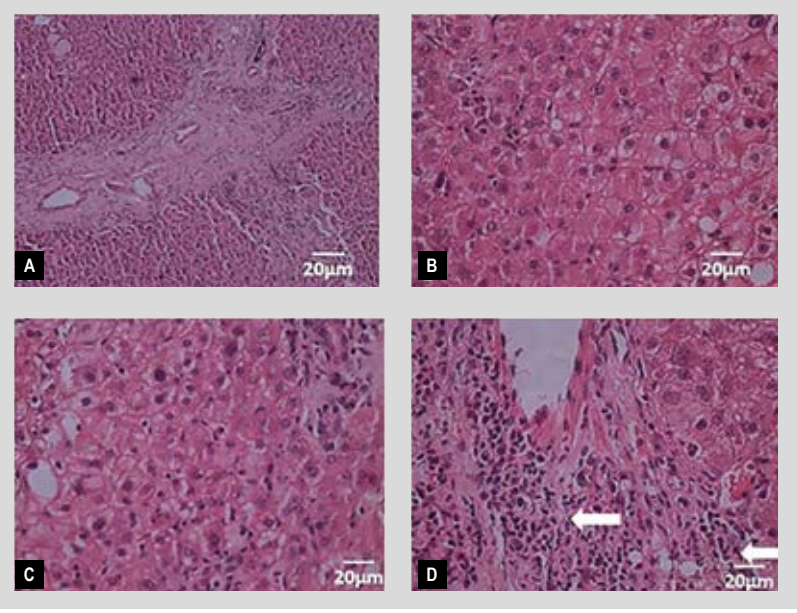

Figure 1. Hematoxylin and Eosin (H&E) dyeing of histologic sections of liver with MAFLD. **A**. Liver tissue with slight increase of connective tissue and inflammatory infiltrate of lymphocyte predominance in the portal space (10x). **B** and **C**. Hepatocytes with abundant cytoplasm slightly eosinophilic and presence of microvesicular and macrovesicular vacuoles (40x). **D**. Arrows indicate inflammatory infiltrate with lymphocyte predominance. Observation made using a 40x objective.

of TWEAK, HSP70 and HSP90 in MAFLD, we performed immunohistochemistry and Western blotting for Fn14.

Anatomohistopathologic analysis of liver tissue with steatosis

The histopathological study of the liver samples studied was conclusive for microvesicular steatosis. In 10% of the samples, an inflammatory infiltrate of lymphocyte predominance was found (Figure 1).

Immunohistochemical analysis of TWEAK-Fn14 in steatotic liver tissue and Immunoreactivity with cellular stress markers

The expression of the cytokine TWEAK and its receptor Fn14 in steatotic liver tissue is shown (Figure 2A and 2B). The expression of these proteins in liver tissue affected by lipid accumulation was localized in the cytoplasm of hepatocytes, showing a higher intensity of reactivity for Fn14 compared to its ligand TWEAK. We were able to

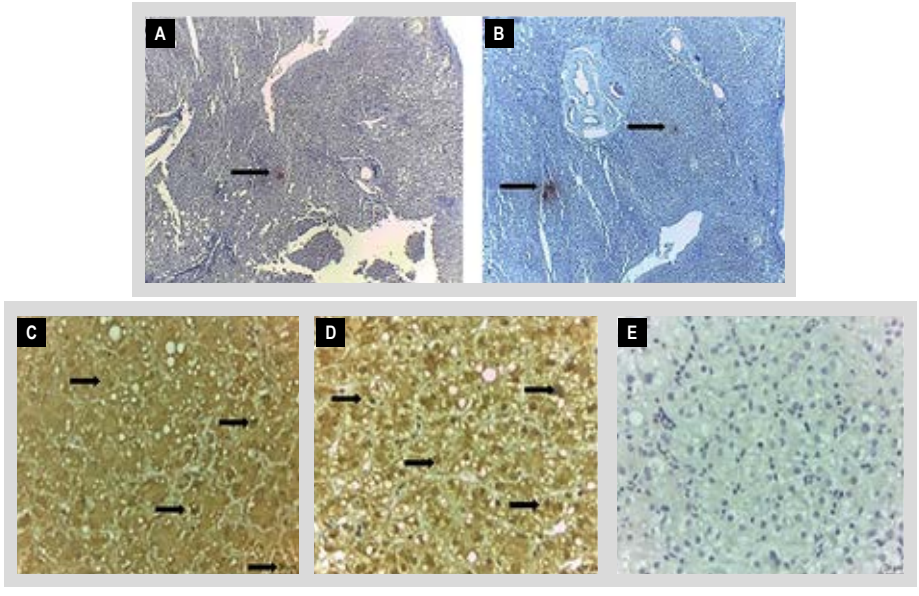

Figure 2. Representative positive immunostaining in MAFLD by immunohistochemistry. **A.** TWEAK protein. **B.** Fn14 receptor and histological sections of steatotic liver representing cellular stress by immunolocalization of HSP70 and HSP90. **C.** Immunoreactivity to HSP70 in cytoplasm and nuclei of liver cells. **D.** Liver cells positive for the cellular stress marker HSP90. In **C** and **D**, lipid deposits are clearly observed at the cytoplasm level. **E.** Immunohistochemical control slide. Observations made at 40X.

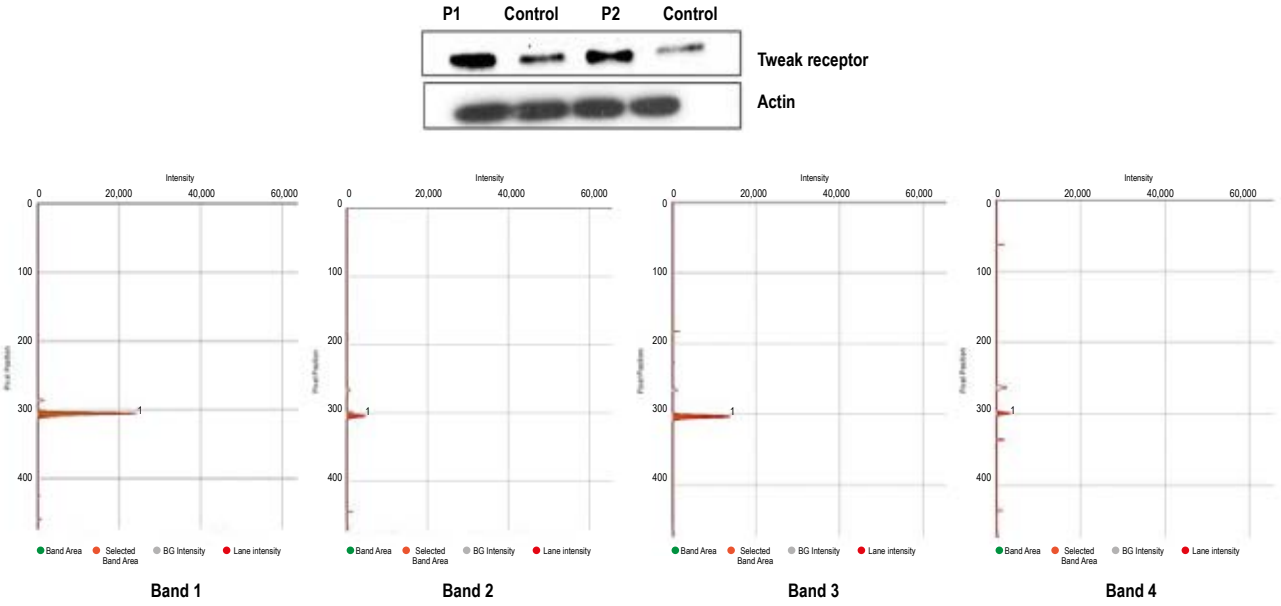

Figure 3. Expression and quantification of Fn14 in hepatic steatotic samples. Analysis performed by western blot, using FN14 antibody 1:1000. Much more marked expression was observed in the hepatic samples with steatosis (MAFLD P1 and P2) with respect to the controls.

observe liver steatosis and consequent activation of cellular stress, as requirements for activation of liver regeneration processes. Immunohistochemical analysis of steatotic liver tissue revealed a high level of expression of heat shock proteins 70 and 90 in the hepatocytes evaluated. In addition, lipid infiltration was observed in the liver tissue, pointing to the pathological condition of MAFLD (Figure 2C and 2D). These findings point to increased cellular stress in this clinicopathological condition.

Western blot analysis of Fn14 in steatotic liver tissue

Tissues with steatosis analyzed corresponding to MAFLD showed positive modulation of protein expression of the Fn14, relative to liver tissue without steatosis (Figure 3).

DISCUSSION

MAFLD is a public health problem that during the current SARS-Cov-2 pandemic has been a challenge for specialists, since it has been observed that this condition affects the final outcome in COVID-19, as well as increasing the probability of becoming severe ill [13]. Likewise, patients with a certain degree of steatosis present serious alterations of several genes related to hepatocarcinogenesis [14]. Therefore, studies that help us to understand the behavior of steatotic liver tissue through biochemical studies are of great importance to propose targets that help to intervene in the disease. Various signals are involved in liver regeneration, such as HGF/cMet, which has been shown to be involved in inducing progenitor cell migration in murine models, as well as the TWEAK/Fn14 pathway [6,15,16]. In this study, we evaluated the behavior of TWEAK/Fn14 axis, as well as cellular stress signals as biochemical indications of a possible liver regeneration in conditions of tissue damage caused by excessive lipid accumulation. The steatotic liver tissues evaluated correspond to individuals with an average age of 42 years; population characterized by a high prevalence of chronic non-communicable diseases, which are associated with the development of MAFLD, which corresponds to the findings of Lambis *et al.* in a Colombian Caribbean population [17,18].

In the immunohistochemistry of the steatotic tissue, a polymorphonuclear infiltrate (lymphocytes) was observed, which could be part of an inflammatory component, and abundant connective tissue around the portal space, which possibly indicates that a fibrotic process is beginning that can be reversible. Hepatocellular damage caused by lipid accumulation usually presents as a degenerative lesion in which the hepatocyte increases considerably in size and takes on a rounded shape. The cytoplasm appears transparent, with a reticulated appearance or with the organelles grouped around the nucleus, which is known as balloon degeneration, ballooning or hydropic degeneration and is due to an accumulation of water and proteins in the cytoplasm of the hepatocyte [19]. Fatty liver tissue has been associated with cytokine production and macrophage accumulation, hepatocyte death and active response of progenitor cells, as a mechanism to direct tissue regeneration [20,21].

In this study we demonstrated positive expression of Fn14 and TWEAK proteins and heat shock proteins in steatotic

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liver tissue. Heat shock proteins 70 and 90 confirm the findings of other studies suggesting the inflammatory environment and stress of fatty liver cells; it has also been observed in murine models that overexpression of HSP90 increased the accumulation of neutral lipids, exerting an important role in MAFLD [22]. In liver injury caused by excessive lipid accumulation, the liver responds by initiating an inflammatory response, which allows the dual action of the resolution of such injury, through the delayed initiation of regeneration of the injured tissue comprising the activation and differentiation of hepatic progenitor cells (HPC) [23,24]. TWEAK together with its surface receptor Fn14 has been described to be significantly involved in the activation and proliferation of HPC and in the restoration of liver mass following hepatocyte damage [2,25,26].

The positive expression of the Fn14 receptor in the tissue and the TWEAK protein confirm what has been suggested by studies in murine and cellular models, which may be related to the severity and progression of steatosis and with the possible activation and proliferation of liver progenitor cells in healthy liver undergoing partial hepatectomy and under pathological conditions [5,6,27]. It has also been proposed in other studies that the intensity of Fn14 expression is parallel to the progenitor and fibrotic response [28]. According to the results found in this study, possible damage at the cellular level produced by liver steatosis can be seen. The expression of the cytokine Fn14 in steatotic liver tissue suggests the possible activation of the TWEAK/Fn14 signaling pathway in MAFLD, highlighting its importance in the process of restoration of liver mass following liver parenchymal damage caused by excessive lipid infiltration. However, further investigations are needed to confirm these findings in prospective studies and to elucidate the possible mechanisms of these associations.

It's important to emphasize that a significant limitation of our study lies in the limited number of samples and healthy tissue available for comparison. This is due to the nature of sampling and the difficulty in obtaining such samples. Although we have observed a positive expression of TWEAK/Fn14 in steatotic tissue, comparing with a greater number of samples of healthy hepatic tissue will provide a better understanding of the TWEAK/Fn14 pathway in the regeneration process in the condition of hepatic steatosis. Therefore, we acknowledge that the interpretation of our results could benefit from a broader comparison with samples of healthy hepatic tissue in future studies.

In conclusion, the expression of the cytokine Fn14 in steatotic liver tissue suggests the possible activation of the TWEAK/Fn14 signaling pathway in MAFLD, highlighting its importance in the process of restoration of liver mass following liver parenchymal damage caused by excessive lipid infiltration. However, further investigations are needed to confirm these findings in prospective studies and to elucidate the possible mechanisms of these associations.

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