Research Note: Expression of IGF-1 and IGF-1 receptor proteins in skeletal muscle fiber types in chickens with hepatic fibrosis

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ABSTRACT We investigated the expression of insulin-like growth factor 1 (**IGF-1**) and IGF-1 type 1 receptor (**IGF-1R**) in skeletal muscle fiber types in chickens with hepatic fibrosis induced by bile duct ligation (**BDL**). Eleven hens, approximately 104 weeks old, were randomly assigned to BDL (n = 4) and sham surgery (SHAM; n = 7) groups. In BDL hens, histopathology revealed marked bile duct proliferation and liver fibrosis. The cross-sectional area (**CSA**) of myofibers from both the pectoralis (**PCT**) muscles significantly decreased in the BDL group compared with the SHAM group ($P < 0.01$). In contrast, the CSA of myofibers from the femorotibialis lateralis (**FTL**) muscle did not decrease in the BDL group. Type I fibers were large, round, and hypertrophic. Elongated type IIA and IIB fibers were also present. For IGF-1 immunostaining, the immunoreaction intensity was higher in the PCT in the BDL group than the SHAM group. Within the BDL group, type I fibers from FTL had a stronger immunoreaction intensity than the type II fibers. For IGF-1R immunostaining, the intensity of the immunoreactions was similar within the PCT in the BDL group compared with the SHAM group. For FTL, type I fibers had stronger reactions to IGF-1R than type II fibers in the BDL group. These results suggest that type I fibers express both IGF-1 and IGF-1R and become hypertrophic in chickens with hepatic fibrosis.

Key words: hepatic fibrosis, fiber types, hypertrophy, IGF-1, IGF-1 receptor

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INTRODUCTION

Skeletal muscle atrophy is a common and serious complication in patients with hepatic fibrosis/cirrhosis (Vural et al., 2020). Till date, experiments investigating the molecular mechanisms behind skeletal muscle atrophy have been performed on rodents using bile duct ligation (**BDL**). Mammalian skeletal muscle is composed of three distinct muscle fiber subtypes, that are defined according to their myosin heavy chain isoforms and metabolic activity. These fiber subtypes include slow-twitch fibers (type I) and fast-twitch fibers (types IIA and type IIB) (Hakamata et al., 2018). In avian species, the M. pectoralis (PCT) in chickens is composed of almost entirely of type IIB fibers (Barnard et al., 1982). The M. femorotibialis lateralis (FTL) consists of a mixture of type I, IIA, and IIB fibers (Suzuki et al., 1985). For this reason, FTL may be appropriate for studying the reaction of type II fibers as well as the interactions among the 3 fiber types.

Insulin-like growth factor-1 (**IGF-1**) is an important hormone in skeletal muscle growth. In many chronic diseases, IGF-1 levels are reduced, and the downstream signaling of IGF-1 type 1 receptors (**IGF-1R**), leading to muscle atrophy (Yoshida and Delafontaine, 2020). Few reports have investigated the response of different fiber types to IGF-1 in hepatic fibrosis/cirrhosis. The action of IGF-1 is mainly facilitated by IGF-1 type 1 receptors (Martin et al., 2018). IGF-1 is primarily synthesized and released by hepatocytes, but skeletal muscle cells produce IGF-1 as well (Yoshida and Delafontaine, 2020). Information regarding the localization of IGF-1 in different skeletal muscle fiber types is limited.

In our previous work, we focused on the atrophy of different muscle fiber types following BDL in chickens. We concluded that hepatic fibrosis, as a result of BDL surgery induced skeletal muscle atrophy in PCT type II fibers (Nagasao et al., 2021). In FTL, type I fibers became hypertrophic, but atrophy was observed in type II fibers (Nagasao et al., 2021). Further investigation is

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required as to why hypertrophy only occurs in type I fibers. Furthermore, interactions among the 3 fiber types also merits further consideration.

Skeletal muscle atrophy, as a result of hepatic fibrosis, is not problematic in poultry production. The effects of hepatic fibrosis and cirrhosis on skeletal muscles have been demonstrated in mammals, such as rats and mice. However, since the skeletal muscles of chickens are larger, have a specific composition of fiber types (i.e., type IIB in PCT), we opted to use chickens in our study (Suzuki et al., 1985).

In this study, we used immunohistology to investigate the localization of IGF-1 and IGF-1R in different muscle fiber types. This was carried out to determine whether IGF-1 and IGF-1R mediate interactions between the different skeletal muscle fibers in chickens with hepatic fibrosis. This research might lead to an understanding in the morphological changes in chickens.

MATERIALS AND METHODS

Animals

This protocol was approved by the President of Kitasato University through the Institutional Animal Care and Use Committee of Kitasato University (approval no. 20-211). Eleven Julia light hens were purchased from a commercial farm at approximately 104 wk old (Yamashoufoods Co., Ltd., Towada, Aomori, Japan). All hens were bred in separate cages and given a commercial diet. Food and water were available ad libitum.

BDL Model

After 7 d of acclimatization to their new environment, 7 and 4 hens were randomly assigned to the SHAM and BDL groups. BDL and sham surgery were performed after 12 h of fasting and water deprivation. Briefly, all hens were anesthetized using isoflurane inhalation. In the BDL group, the common hepatoenteric and cysticoenteric ducts were isolated, and double ligation was performed. In the control hens, both bile ducts were exposed, but double ligation was not performed. Following these procedures, all hens were injected intraperitoneally with penicillin G (1,000,000 U, Meiji Seika Pharma, Tokyo, Japan) to prevent bacterial infection. The abdominal walls were closed. After surgery, the hens were returned to their separate cages for recovery. All hens were maintained for 4 wk after surgery and had access to food and water ad libitum.

Tissue Preparation

Four weeks after surgery, all hens were anesthetized by an intravenous injection of sodium pentobarbital (150 mg/kg) into the wing vein and euthanized. Liver samples were collected, fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin wax. Tissue sections (4-μm thick) were cut and stained with hematoxylin and eosin (**HE**) for histological examination. The PCT and FTL muscles were excised and samples from the mid-regions of these muscles were trimmed into blocks, (approximately 1.0 cm × 0.5 cm) and mounted on cork boards using 10% tragacanth gum (Nacalai Tesque, Kyoto, Japan). The muscle tissues were placed in a specific orientation so that the fibers could be cut transversely. The blocks were frozen by immersion in isopentane and, freezing in liquid nitrogen, and stored at −80°C until further analysis. For morphological examination and classification of fiber types, serial cross-sections of 10 μm were cut using a cryostat microtome (Leica Biosystems, Tokyo, Japan) at −20°C. Each serial cryosection was mounted on MAS-coated glass slides (Matsunami Glass, Osaka, Japan) and maintained at −20°C.

Histology and Fiber Typing

The HE staining was carried out on the first batch of serial cross-sections for histological examination. The second and third batches of serial cross-sections were used for histochemical identification of type I or II myofibers using the myosin ATPase technique at pH 4.3 or pH 10.5, respectively (Suzuki et al., 1985). The fourth batch of serial cross-sections was used for histochemical identification of types IIA or IIB fibers by employing the nicotinamide adenine dinucleotide dehydrogenase−tetrazolium reductase technique (**NADH-TR**) (Suzuki et al., 1985). The cross-sectional area (**CSA**) was determined by randomly selecting 120 skeletal muscle cells from each hen per slide. The perimeters of those cells were measured using ImageJ 1.52a software (Rasband, National Institutes of Health, Bethesda, Maryland). For fiber typing, 120 skeletal muscle cells assessed using myosin ATPase staining and NADH-TR (Suzuki et al., 1985), were used for type I, IIA, or IIB fibers.

Immunohistochemistry

For the detection of IGF-1 and IGF-1R in skeletal muscles, the fifth and sixth batch of serial cross-section was used. Tissues were fixed in 10% neutralized buffered formalin for 10 min at approximately 23°C. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide diluted in methanol for 20 min. The primary antibodies used in this study included anti-IGF-I antibody, clone Sm1.2 (Merck Millipore, 1:50), and anti-IGF1R/IGF1 receptor rabbit anti-human polyclonal (pTyr1161; LSBio, 1:100). The cross-sections were incubated with the primary antibodies for 12 h at 4°C. As a secondary antibody, peroxidase-conjugated anti-rabbit IgG (MAX-PO [R], Nichirei Biosciences Inc., Tokyo, Japan) or anti-mouse IgG (MAX-PO [M], Nichirei Biosciences Inc., Tokyo, Japan) was used for 1 h at approximately 23°C. The immunohistochemical signal was detected on brown color staining using DAB

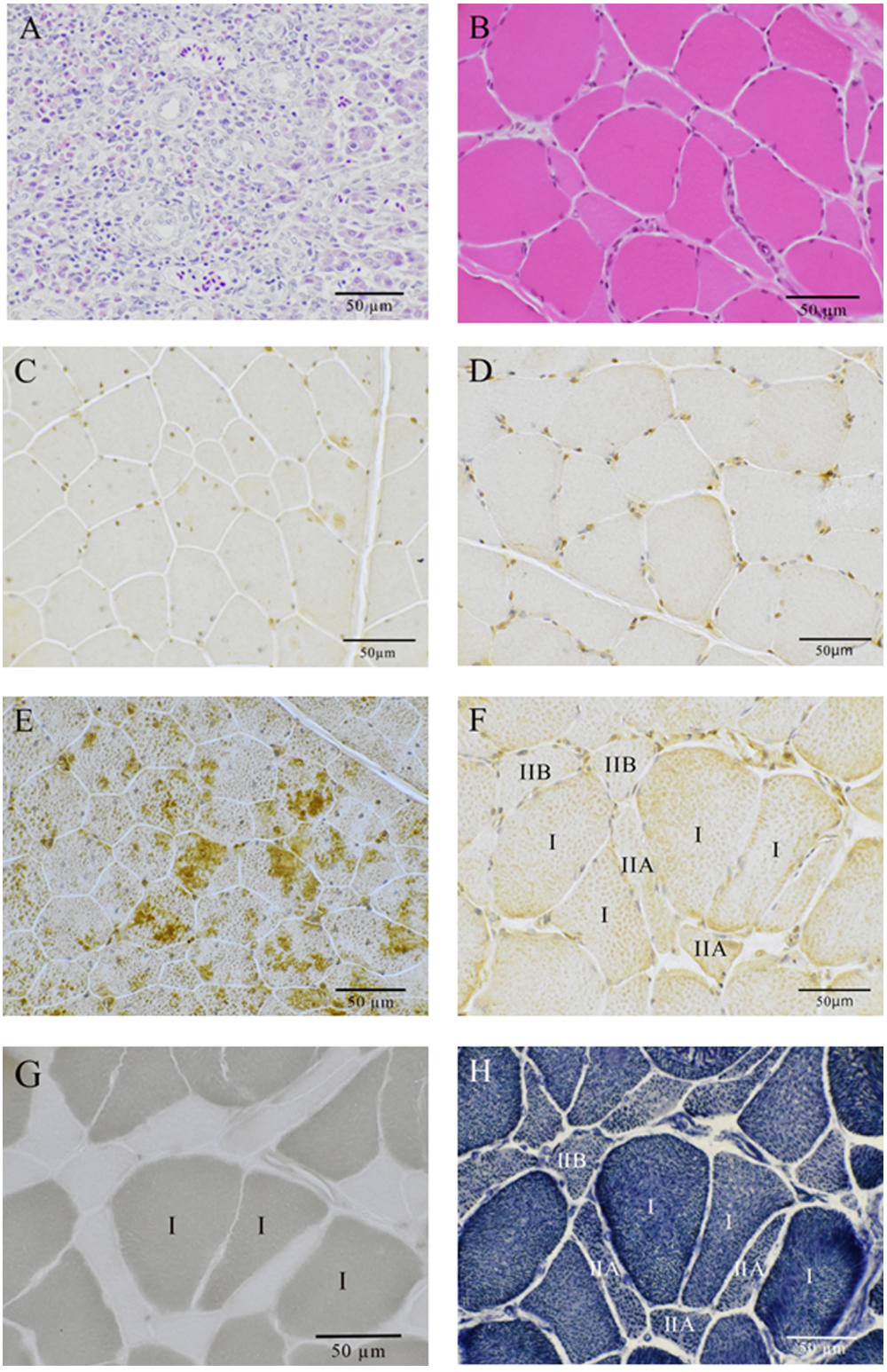

Figure 1. (A) Histological observations of the liver on hematoxylin and eosin (H&E) staining in the bile duct ligation (BDL) group. All hens in the BDL group had yellow/white livers, with numerous white surface nodules and enlarged gall bladders. Histopathology revealed marked bile duct proliferation and fibrosis (A). Bar: 50 μm. (B) Histological observations of femorotibialis lateralis (FTL) on hematoxylin and eosin (H&E) staining in the BDL group. Both large, round myofibers with eosinophilic sarcoplasm and smaller, elongated shaped myofibers are present (B). Bar: 50 μm. (C, E) Immunohistochemical localization of IGF-1 in pectoralis (PCT) myofibers in SHAM and BDL groups. A more intense and diffuse IGF-1 expression was noted in the BDL group compared with the SHAM group. Bar, 50 μm. (D, F, G, H) Immunohistochemical localization of IGF-1 in myofibers in FTL of SHAM and BDL groups. The intensity of IGF-1 expression was similar in type I and II fiber types in SHAM chickens. In contrast with SHAM chickens (D), a higher expression of IGF-1 was noted in type I fibers than in types IIA and IIB fibers in BDL chickens (F). Classification of fiber typing was conducted using myosin ATPase (pH 4.3) for type I fibers or type IIA fibers (G) and NADH-TR for types IIA or IIB fibers (H).

(3,3′-Diaminobenzidine). Nuclei were counterstained with hematoxylin. All images were acquired using a microscope (Eclipse Ci; Nikon, Tokyo, Japan), equipped with a digital camera (D5600; Nikon). As for the quantification of the staining, the intensity of the positive signal was compared between the SHAM and the BDL group, using light microscopy. The intensity of the signal of the SHAM group was regarded as normal and compared with that of the BDL group for each muscle fiber and primary antibody.

Statistical Analysis

Results are presented as mean ± standard error of the mean. Statistical analyses were performed using repeated-measures one-way ANOVA, followed by Tukey-Kramer's test for multiple datasets with Bell Curve for Excel software. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Histological Changes in Skeletal Muscles

Both the gross and histological appearance of SHAM group hen livers were normal. In contrast, livers from hens in the BDL group were yellow−/white. Furthermore, numerous white surface nodules and enlarged gall bladders were visible. Histopathological findings revealed marked bile duct proliferation and fibrosis (Figure 1A). These findings are also observed in BDL hen livers in our previous reports (Nagasao et al., 2021). The myofibers from PCT and FTL in the SHAM group, were polygonal with multiple subsarcolemmal nuclei, and were similar in morphology to all skeletal muscle types. Almost all PCT myofibers from the BDL group were smaller than SHAM group myofibers. The CSA of myofibers from PCT was significantly lower in the BDL group than in the SHAM group ($P < 0.01$; see Table 1). Myofibers from the FTL BDL group were generally large and round, with eosinophilic sarcoplasm. However, small elongated myofibers were also observed (Figure 1B). The CSA of fiber types from FTL, showed a significant increase in type I fibers in the BDL group compared with the SHAM group ($P < 0.01$; Table 2). In contrast, type IIA and IIB fibers significantly decreased in the BDL group compared with the SHAM group ($P < 0.01$; Table 2).

Table 1. Mean cross-sectional area (μm^2) of skeletal muscles in control and bile duct ligation groups.

	SHAM (n = 7)	BDL[a] (n = 4)
PCT[b]	2275.0 ± 300.2	520.0 ± 222.0*
FTL[c]	2363.7 ± 146.2	2239.7 ± 211.4

*Indicates significance at $P < 0.01$.
[a]Bile duct ligation.
[b]Pectoralis.
[c]Femorotibialis lateralis.

Table 2. Mean cross-sectional area (μm^2) of type Ⅰ, ⅡA, and ⅡB myofibers in Femorotibialis lateralis of SHAM and BDL groups.

	SHAM (n = 7)	BDL[a] (n = 4)
Type Ⅰ	1949.6 ± 54.7	2540.1 ± 101.8*
Type ⅡA	1917.1 ± 49.6	919.2 ± 29.1*
Type ⅡB	2724.4 ± 65.7	1203.1 ± 48.6*

*Indicates significance at $P < 0.01$.
[a]Bile duct ligation.

Expression of IGF-1 and IGF-1 Receptor in Each Fiber Type

In the SHAM group, IGF-1 was localized diffusely throughout the sarcoplasm and more concentrated in certain regions under the sarcolemma. Almost all myofibers in the SHAM group were positive for IGF-1 expression in both PCT and FTL (Figures 1C and D). In FTL, positive IGF-1 cells were observed in type I, IIA, and IIB fibers. In PCT, IGF-1 was mainly localized in the sarcoplasm of type IIB fibers in the BDL group. The immunoreactions in both muscles were stronger in the BDL group than in the control group (Figure 1E). In FTL, immunoreactions were stronger in the large, round myofibers, that is, type I fibers, than in type II fibers (Figures 1F−1H).

In the SHAM group, IGF-1R was localized in a concentrated manner under the sarcolemma in both PCT and FTL (Figures 2A and 2B). A combination of both IGF-1R positive and negative myofibers were present in both types of skeletal muscles. In PCT, immunostaining intensity of the positive myofibers was reduced in the BDL group compared with the SHAM group (Figure 2C). In FTL, relatively stronger positive IGF-1R reactions were observed in type I fibers than in type IIA or IIB fibers (Figures 2D−2F).

These results suggested that BDL might strongly induce expression of IGF-1 in atrophied IIA and IIB skeletal muscle, fibers when IGF-1R expression is reduced. IGF-1 production in skeletal muscle varies to regulate muscle growth in a paracrine/autocrine manner via the IGF-1 receptor (Clemmons, 2009). IGF-1R is present in all cell types and tissues, including skeletal muscles (Clemmons, 2009). Oudin et al. also demonstrated a low number of IGF-1R in the breast muscle (type IIB) and leg muscle (type I and IIA) in broiler chickens, aged 1 to 7 wk (Oudin et al., 1998). In this study, it is presumed that the low number of IGF-1R is related to the older age of the hens; however, we propose that IGF-1 might be involved in the hypertrophy of type I myofibers.

The difference in IGF-1 expression intensities, that is, in type IIA or IIB fibers, between PCT and FTL suggest that type II fibers in the FTL synthesize IGF-1 for type I fibers in an autocrine/paracrine manner. There are few reports on IGF-1 expression among fiber types. However, a similar IGF-1 expression pattern was observed in the breast and leg muscles of both males and female chickens, where the leg muscle had relatively higher IGF-1 expression levels than the breast muscle (Abdalhag et al., 2016).

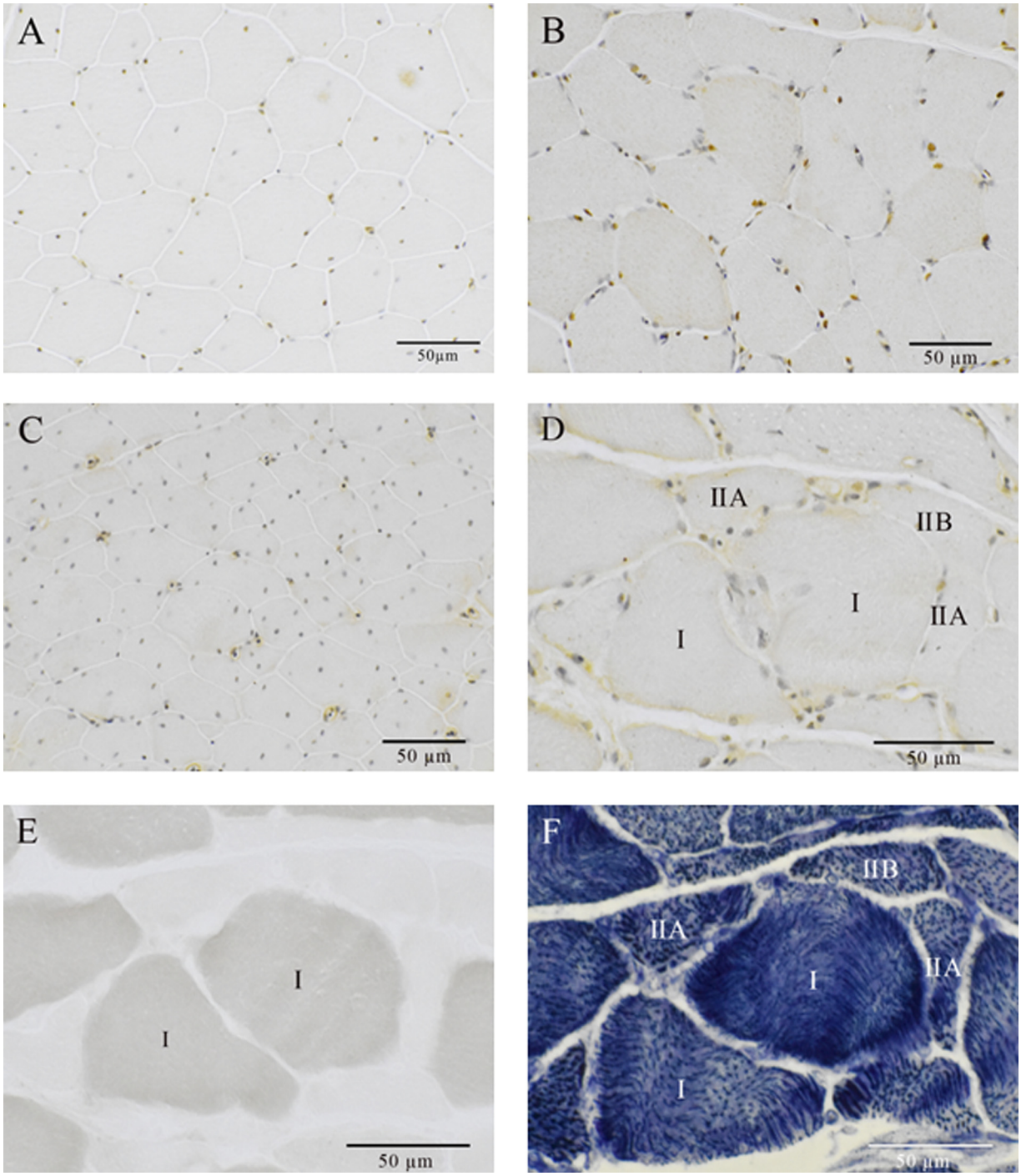

Figure 2. (A,C) Immunohistochemical localization of IGF-1 receptor in myofibers in PCT of SHAM and BDL groups. Positive reactions were observed in the interstitial cells rather than in myofibers in bile duct ligation (BDL) group. (B,D,E,F) Immunohistochemical localization of IGF-1 receptor in myofibers from the femorotibialis lateralis muscle in SHAM and BDL groups. Most myofibers expressed the IGF-1 receptor in SHAM chickens (B). In the BDL group, the positive reactions were mainly observed in type I fibers as opposed to type IIA and IIB fibers (D). Type II fibers were classified using myosin ATPase (pH 4.3) for types I or IIA fibers (E) and NADH-TR for types IIA or IIB fibers (F). Bar, 50 μm.

In this study, it was unclear whether type IIA or IIB fibers synthesized IGF-1 or if the surviving type IIA or IIB fibers in the FTL synthesized IGF-1 for the PCT in the BDL group. IGF-1 expression in mRNA level should be assessed by in situ hybridization technique.

In conclusion, our study suggests that type I fibers in the FTL muscle express both IGF-1 and IGF-1R as a result of hepatic fibrosis and become hypertrophic under these conditions. The knowledge gained with regards to the role that IGF-1 plays among fiber types may lead to a better understanding how skeletal muscles adapt under hepatic fibrosis.

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DISCLOSURES

The authors have no conflicts of interest to report.

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