Acta Biomaterialia 30 (2016) 265–276

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Impaired primary mouse myotube formation on crosslinked type I collagen films is enhanced by laminin and entactin

S. Grefte [1], M.J.W. Adjobo-Hermans, E.M.M. Versteeg, W.J.H. Koopman, W.F. Daamen *

Department of Biochemistry, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

ARTICLE INFO

Article history:
Received 15 July 2015
Received in revised form 16 October 2015
Accepted 6 November 2015
Available online 7 November 2015

Keywords:
ECM (extracellular matrix)
Muscle
Myotubes
Collagen
Laminin

ABSTRACT

In skeletal muscle, the stem cell niche is important for controlling the quiescent, proliferation and differentiation states of satellite cells, which are key for skeletal muscle regeneration after wounding. It has been shown that type I collagen, often used as 3D-scaffolds for regenerative medicine purposes, impairs myoblast differentiation. This is most likely due to the absence of specific extracellular matrix proteins providing attachment sites for myoblasts and/or myotubes. In this study we investigated the differentiation capacity of primary murine myoblasts on type I collagen films either untreated or modified with elastin, laminin, type IV collagen, laminin/entactin complex, combinations thereof, and Matrigel as a positive control. Additionally, increased reactive oxygen species (ROS) and ROCK signaling might also be involved. To measure ROS levels with live-cell microscopy, fibronectin-coated glass coverslips were additionally coated with type I collagen and Matrigel onto which myoblasts were differentiated. On type I collagen-coated coverslips, myotube formation was impaired while ROS levels were increased. However, anti-oxidant treatment did not enhance myotube formation. ROCK inhibition, which generally improve cellular attachment to uncoated surfaces or type I collagen, enhanced myoblast attachment to type I collagen-coated coverslips and -films, but slightly enhanced myotube formation. Only modification of type I collagen films by Matrigel and a combination of laminin/entactin significantly improved myotube formation. Our results indicate that type I collagen scaffolds can be modified by satellite cell niche factors of which specifically laminin and entactin enhanced myotube formation. This offers a promising approach for regenerative medicine purposes to heal skeletal muscle wounds.

Statement of significance

In this manuscript we show for the first time that impaired myotube formation on type I collagen scaffolds can be completely restored by modification with laminin and entactin, two extracellular proteins from the satellite cell niche. This offers a promising approach for regenerative medicine approaches to heal skeletal muscle wounds.

1. Introduction

Skeletal muscle has a remarkable ability to regenerate itself after injury [1–3]. Key players in skeletal muscle regeneration are the satellite cells, which reside next to the myofiber beneath the basal lamina [4,5]. Upon injury, satellite cells enter a myogenic program which consists of a proliferation phase where they produce progeny called myoblasts, which then differentiate and fuse to form new, or repair damaged, myofibers [1–3]. A small fraction of the satellite cells self-renew to replenish the quiescent satellite cell pool for future regeneration events [6–8]. When large parts of skeletal muscle tissue are lost, the intrinsic regenerative capacity is not able to efficiently repair the injury and hence scar tissue is formed [2,3,9,10]. To improve skeletal muscle regeneration, several approaches exist such as the addition of growth factors, satellite cells or other cell types [11,12]. However, these approaches are not sufficient to induce skeletal muscle regeneration after tissue ablation. Tissue-engineered constructs might be one of the solutions to fill up the defect and provide structural cues for the

* Corresponding author.
E-mail addresses: sander.grefte@wur.nl (S. Grefte), Merel.Adjobo-Hermans@radboudumc.nl (M.J.W. Adjobo-Hermans), Elly.Versteeg@radboudumc.nl (E.M.M. Versteeg), Werner.Koopman@radboudumc.nl (W.J.H. Koopman), Willeke.Daamen@radboudumc.nl (W.F. Daamen).
[1] Current working address: Department of Human and Animal Physiology, Wageningen University, Wageningen, The Netherlands.

http://dx.doi.org/10.1016/j.actbio.2015.11.009
1742-7061/

satellite cells or myoblasts. Type I collagen is the most abundant extracellular matrix (ECM) protein within mammalian tissue and shows favorable results in promoting wound healing [13–15]. Implantation of type I collagen scaffolds seeded with C2C12 cells improved muscle regeneration [14,16,17], although host cells remained in the outerparts of the construct [17]. However, in some cases type I collagen scaffold failed to improve muscle regeneration [9,18]. Other materials such as decellularized tissue or hydrogels were in some cases not able to enhance muscle regeneration [19–21], but the addition of myogenic cells generally improved the outcome [22–27]. Thus the addition of satellite cells to the scaffolds improved muscle regeneration, however, cultured satellite cells lose their myogenic capacity and mostly die after transplantation [8,28,29]. Addition of growth factors [27,30] or freshly isolated satellite cells improves cell survival [8], but still requires specific culturing conditions [31]. This can be avoided by using scaffolds that attract endogenous cells showing myogenic potential such as CD133+ cells [32] and satellite cells [9]. In the latter study, satellite cells were actively recruited to the muscle wounds by implanting type I collagen scaffolds loaded with stromal-derived factor (SDF)-1α. However, cells did not enter the type I collagen matrix. Moreover, in 2D and 3D cultures of type I collagen, myoblasts lose their myogenic capacity and only form small and rounded myotubes [33]. This suggests that type I collagen does not support satellite cell attachment and therefore myoblast differentiation and fusion. However, we still aim to use type I collagen-based biological materials as this is the main ECM protein found in tissues and one that can be isolated and purified in quantities needed for the production of scaffolds. Therefore our goal is to modify the surface of these scaffolds to achieve optimal myotube formation either by the addition of ECM proteins of the satellite cell niche, which consists mainly of laminin, type IV collagen, type VI collagen, entactin, and perlecan [34,35], or by the addition of pharmacological agents.

Many of the satellite cell niche factors are also found in Matrigel, which is often used as a substrate for *in vitro* culture and has been shown to be more efficient to induce myotube formation compared to type I collagen [33,36]. Of these ECM proteins, laminin binds the integrin α7β1 receptor, which is mainly expressed by satellite cells. In contrast, differentiating satellite cells or myoblasts increasingly express the integrin α3β1 receptor which binds to entactin. Interestingly, myoblasts and myotubes were not able to bind to Matrigel in the presence of antibodies directed to laminin and entactin, respectively [37]. To our knowledge this is the only study showing an important role of entactin in myotube attachment. The significance of using other niche factors has been demonstrated by the fact that laminin is able to increase myoblast adhesion, differentiation and fusion compared to type I collagen [36,38–40]. However, laminin or type IV collagen by themselves are not as efficient in myotube formation as Matrigel [41]. This suggests that combinations of the satellite cell niche factors are more successful in promoting myotube formation compared to niche factors alone or other ECM proteins. Still, another reason might be the presence of certain growth factors (*i.e.* FGF-2, HGF, IGF-1) in Matrigel that stimulate myoblast proliferation and differentiation. In addition to the satellite cell niche factors, elastin also successfully improved myotube formation of C2C12 myoblasts [42] and H9c2 cardiomyoblasts [43] and is therefore promising as well.

Next to the use of ECM-proteins, we also explored the possibilities of using pharmacological agents that target reactive oxygen species (ROS) or the activity of Rho-associated, coiled-coil containing protein kinases (ROCK) to improve myotube formation. It has been shown that ECM influences the production of ROS [44], which modulate myoblast differentiation and fusion [45–47]. Moreover, RhoA, which belongs to the Rho-GTPases, play key roles in skeletal myogenesis [46–49] and cell adhesion [50–53]. Increased RhoA activity has been shown to inhibit myoblast fusion and inhibiting ROCK activity, which are the downstream targets of RhoA, improved myotube formation [54–56]. Additionally, pharmacological inhibition of ROCK activity improved *in vitro* cell attachment of pluripotent stem cells to uncoated surfaces [50], human tenon fibroblasts to type I collagen [51], trabecular meshwork cells to fibronectin and type I collagen [52], and human corneal endothelial cells to fibronectin [53]. Thus, pharmacological agents influencing these processes may also improve satellite cell attachment and differentiation on type I collagen scaffolds.

Taken all together, this urged us to investigate whether type I collagen-based materials can be modified to promote myoblast attachment, differentiation, and fusion and thus be used for regenerative medicine purposes to heal skeletal muscle wounds. To achieve this, the type I collagen films are modulated by coatings of growth factor-free ECM proteins, especially laminin and entactin, which has not been used previously, or the addition of pharmacological agents.

2. Methods

2.1. Animals and housing conditions

Wild-type (WT) mice bred with a mixed 129/sv × C57BL/6 background received a standard rodent diet *ad libitum*. Animals were housed at 21.0 °C and 60% humidity and with a light/dark (12 h/12 h) cycle. All breeding and experiments (RU-DEC2008-080) were approved by the Animal Experimentation Committee at the Radboud University Medical Centre, in accordance with Dutch laws and regulations regarding animal experimentation.

2.2. Collagen-based films

Insoluble type I collagen fibrils purified from bovine achilles tendon was applied [57]. Solubilized elastin obtained by oxalic acid hydrolysis from insoluble elastin fibers, purified from equine ligamentum nuchae, was used [58–60]. Films were prepared composed of collagen only and collagen with solubilized elastin in a 4:1 ratio [61]. Briefly, a suspension of 0.8% (w/v) collagen was made for collagen films and 0.64% (w/v) collagen with 0.16% (w/v) solubilized elastin for collagen-solubilized elastin films and homogenized. Air-bubbles were removed from the suspensions by centrifugation at 250*g* for 10 min at 4 °C. 12 ml of the suspensions were poured into a petri dish (∅ 86 mm) and air-dried at room temperature. The films were chemically cross-linked for 3 h with 33 mm 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and 6 mm N-hydroxysuccinimide (NHS) in 50 mm 2-morpholinoethane sulfonic acid (MES, pH 5.0) containing 40% ethanol. After crosslinking, films were washed and air-dried [61,62]. All films were sterilized by gamma irradiation (25 kGy; Synergy Health, Ede, the Netherlands).

2.3. Myofiber and primary myoblast isolation

Female WT mice (8–12 weeks) were sacrificed and *extensor digitorum longus* (EDL) muscles were carefully dissected. Individual myofibers were isolated as described in detail elsewhere [63,64]. Isolated myofibers were plated onto 6 wells-plates coated with 1 mg/ml Matrigel (Matrigel™ Basement Membrane Matrix, BD Bioscience, Bedford, MA, USA) and cultured with 6 ml DMEM-HG culture medium supplemented with 30% (v/v) fetal bovine serum (FBS, PAA Laboratories), 10% (v/v) horse serum (PAA Laboratories), 1% (v/v) chick embryo extract (MP Biomedicals Europe, Illkirch Cedex, France), 10 ng/ml fibroblast growth factor 2 (FGF-2; Invitrogen), and 1% (v/v) penicillin/streptavidin (PAA Laboratories)

ID	Title	Pages
192	Impaired primary mouse myotube formation on crosslinked type I collagen films is enhanced by laminin and entactin	12