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Evaluation of morphological evidence of Telocyte influence during skeletal muscle formation in rat using anti-CD34

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Abstract:

The word "telocyte" was first used in the scientific press a few years ago to refer to a "new" cell type that Popescu and Faussone-Pellegrini had discovered in the connective tissue of various organs (2012). Since then, 368 papers have been published that use the term "telocyte," 261 of them in the last five years. These figures highlight the scientific community's rising interest in this cell type and the widespread acceptance of the term "telocyte" to describe this interstitial cell.

Many tissues and organs from diverse species have TCs found in them Because TCs play a crucial role in tissue creation, repair, and regeneration as interstitial cells that help putative stem and progenitor cells in stem cell niches in a variety of tissues and organs, it is imperative to fully understand the role of these cells in muscle embryogenesis. Features of immunohistochemistry Recent years have seen the identification and isolation of myogenic cells based on the expression of the CD34 surface receptor, which has historically been utilized as a marker of hematopoietic stem cells.

Currently, many TCs-specific markers have been proposed.

Considerably, CD34 is the most consistent and widespread marker for a variety of progenitors. In this work, we will employ the abcam Anti CD34 polyclonal antibody, a membrane protein that was first discovered on hematopoietic stem cells and facilitates cell-cell adhesion.

Study's purpose of the current study was for better understand how Telecytes cells contribute to the development of skeletal muscle during the embryonic period of myogenesis, this study design enrolled three groups: The groups of animals are divided to 3 groups Group A: 20 Embryos in their 1st week of gestation. Group B: 20 Embryos in their 2nd week of gestation. Group C: 20 Embryos in their 3rd week of gestation.

paraffin block was prepared & evaluated histologically using NDS, CD34 antibodies, and immunohistochemically using the same tools. Analyzing immunohistochemical reactivity was evaluated using the Aprio Image Scop Program, and statistical analysis for the gathered data was done using the ANOVA test and SPSS.

Keywords:

Telocyte influence; skeletal muscle; anti-CD34

Telocytes (TC) were only recently identified, their history is rather recent. But from the start, the expansion of information about TC has been exponential. Interstitial Cajal-like cells, first reported by L.M. Popescu's team from Bucharest in 2005 (Gherghiceanu et al., 2005) the stromal cell type that

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resides in various tissues (ICLC). Considering their obvious resemblance to standard The intestinal pacemaker cells are called interstitial cells of Cajal (ICC), this group called these cells ICLC (Faussone-Pellegrini,2005). in 2008, M.S. Faussone-Pellegrini and her crew from Florence, Italy provided a description of ICLC in the muscular the human gut's exterior and observed diverged Ultrastructure and immunophenotype data from the ICC (Pieri,2008). "novel" existence identifying this odd cell form using immunohistochemistry and electron imaging in the stroma of various organs. They also agreed name ICLC needed to be changed to something more fitting. From that point on, this unique cell type was referred to as the telocyte (Popescu & Faussone-Pellegrini., 2010).

Telocytes are cells that are found various tissues and organs in the interstitial space. respiratory system tract, brain, digestive system, skin, eye, skeletal muscle, digestive glands, urinary tract, vasculature, bone marrow, and other body systems are all located in the heart (in the interstitial areas of the epicardium, myocardium, and endocardium). Telocyts play a variety of activities in the body, such as organizing connective tissue, thanks to their capacity to create three-dimensional networks. control of immunological reactions, assist or nurture stem cell niches in a body contain potential stem and progenitor cells. variety of organs and body parts, and maintain homeostats and intercellular signaling. Currently, many TCs-specific markers have been proposed. CD34 polyclonal antibody will be used in this investigation.

Experimental Animals and Housing

After mating, female rat vagina was checking for the presence of vaginal plug (a whitish mass of seminal fluid composed from the male mouse that filled the vagina for 8 - 24 hours) and after fertilization separated the pregnant rat was from the male and that day represented day zero of pregnancy, The gestation period length is about (19-21) days. Embryos were collected according to gestational age. Animals were scarified using cotton wool soaked in chloroform for 5–10 minutes in an airtight chamber. The animal was then placed on its back and secured to the dissection table using its four limbs. With a knife and scissors, the abdominal wall was dissected.

A ventral midline incision was made in the abdomen, the skin was pulled back on the sides, the peritoneal cavity was opened and exploration of the abdominal viscera done looking for the abdominal cavity of pregnant females after (5,7,9,11, 12,13,14, 15,16,19,21) day. Embryos were collected according to their gestational age, washed after their extraction from the uterus with normal saline, then after washing the embryos they were immediately fixed in 10% formalin for 48 hours.

The sample became ready for histological preparation of dehydration, clearing, impregnation, embedding, sectioning, 5 micron section was laid on positively charged slide ready for IHC, Plastic section was processed using araldite and then the o.5micron section was stained with Nuclear differentiation special stain (NDS)

NDS stain is composed of two solutions: Solution A: Basic fuchsine & methanol, Solution B: Prepared by mixing equal volumes of Azure II, Methylene blue, Na2Co3 & Absolute methanol alcohol was based on modification and a combination of methods suggested by (Alhabib,.2000).

Immunohistochemical reaction:Rabbit polyclonal to CD34 Antibody potent mitogen was used in this study, its dimerization polyclonal rabbit antibody provided in liquid form shipped at 4ºC, PH7.4(Code number: ab81289).

Result: Nuclear differentiating stain allow to examine the cells closely and thoroughly being based on very thin sections (0.5 µm) which give this stain the criteria. Area surrounding bones were examined at first week of gestation showed many small size myoblasts in between mesenchymal connective tissue.

Myoblasts also Known as (myogenic progenitors' cells of mesoderm) are small cells with basophilic cytoplasm centrally located nuclei, the cells usually have irregular outline & shape, but its shapes more rounded than other mesenchymal cells with abundant cytoplasm Mesenchymal cells are elongated with cytoplasmic process figure(1).

With the advancement of gestational age and around the beginning of the second week of gestation, myoblast started to gain increase the size and length. This stage characterized by the appearance of different

form of cells together, with the advancement of maturation and differentiation of myoblast. The myoblast started to gain maturity first by gaining more cytoplasm then by starting to elongate and form myocytes. A striking feature is that there are different forms of the same cells at the same time where Myoblast, Myocytes & early myotube was identified at the same section figure(2).

Myocytes start to gather & elongate with gaining more cytoplasm & still they have centrally located nuclei. as a preparation for fusion and forming myotubes. New small myotubes will be formed these myotubes will Start to form groups in between which mesenchymal tissue is still present figure(3).

The field seems to be occupied by different types of cells that were different in their nuclear appearance large cells with large centrally located nuclei were seen representing the growing myocytes multiple other nuclei representing all. types of connective tissue cells (fibroblast, mesenchyme cell, macrophages and stellate cell). One type of nucleus which was seen surrounding the periphery with growing myoblast with its unique very long slim type of nucleus. Which could be most probably represents telocyte figure(4)& figure(5).

At the same time that growing myoblast and new formed myotube start to form groups start to form bundles. Toward the end of 2nd week of gestational age, different form maturity of skeletal muscle was seen at the same time where we can see new myotubes with centrally located train nuclei and the start of striation appearance will either cell start to gain more maturity features septically peripherally located nuclei figure(6) & figure(7).

Assessment of different types of cell nuclei occupying the scene showed a specific type of very long nucleus which is usually appear as flattened compressed with densely chromatin appearance found in between the growing muscle cells specifically in the third gestation group of only their cytoplasmic appearance showed very long thin processes extending between skeletal muscle cells which most probably represent telocytes figure(8).

Polyclonal CD34 antibody was used to demonstrate Telocyte reactivity for the antibody in different gestational age groups. The reactivity expression was demonstrate as brown colour reaction in different grades, this grades reanged very faint brown color to dark brown color. CD34 expression in first week age groups was very difficulte to be identified, localazation of the reaction was investigated at the area around the bone were future muscle cells should be located. The field showed non or very faint reaction examination of the same field Using Aprio image scope Software showed few point of expression as yellow marked areas (weak Positive)

assasment of the second week of gestational age reactivity toward CD34 showed a different view than what have been seen in the first week age group. well identified area selected also around the bone showed will Identified brownish colouration. were newly formed muscle cells Should be located. Assessment of this expression using Aprio image scop Software showed heavy reaction ranged from orange brown to red brown colour. Some areas showed heavy expression of CD34 than other Areas.

In third week, the expression of CD34 were notes to be less than what have been seen in the second week although it was still confined to area around or nearby bone muscle at this age. CD34 expression was mainly and easily identified only the periphery of the muscle bundles. Higher magnification of the area showed intense dark brown colouration of the periphery of the muscle bundle,not all muscle bundle showed this feature. Statistical analysis of Immun histo chemical expression: CD 34 expression in different groups was statistically assessed & the mean value of positive pixels was evaluted using Aprio Image Scope software for all groups involved in this study. Immun histochemical reactivity expression of CD 34 for 1st week group was. (164738.67 ±24434.1) pixles / μ^2. while 2nd week group recorded an expression of (290873.84 ± 26813.4) pixles / μ^2 There was significant difference between the 2 groups.

were (98 389.8 ± 16.395) Pixle /μ^2. Comparisons between 2nd week group & third week group showed significant difference with P value of < 0.001. Comparison between 1st week & third week CD34 expression assessed by Aperio Software showed that there is no significant differences between them with a p Value of 0.29. Differences between the 3 groups enrolled in this study can be assed together in table 1 & figure (9) were P_1 / P3 was significant & p2 was Non-significant.

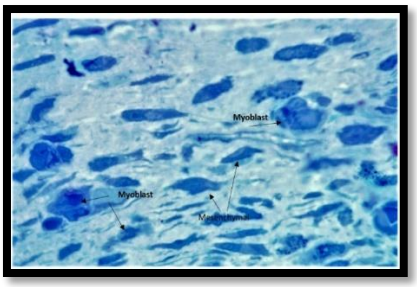


Figure 1: section showing mesenctymal tissue arround bons with different types of cells Myoblast canbe seen as round centerally located nucleans cells (M). 1st week, NDS, X 40

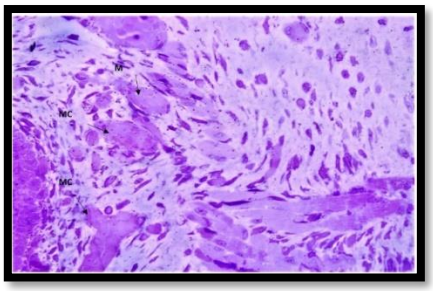


Figure 2: Myocytes (MC) gain of more cytoblast & elongation. 2nd week ,NDS, X 100

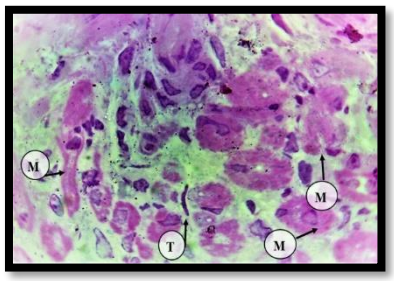


Figure 3: Section showing multiple Myocytes (MC) arranging themselvg with telocytes (TS) ,2nd week, ,NDS, X 100.

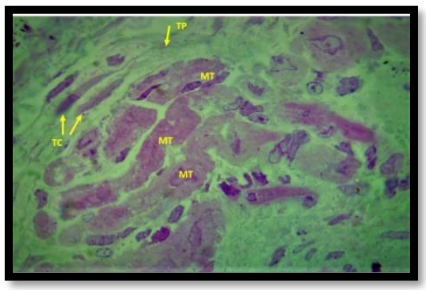


Figure 4: section showing formation of new myotube (MT) with many telocyte adjecent to it (TC) , telopods are seen (TP) , extending form the cell, NDS, X 100

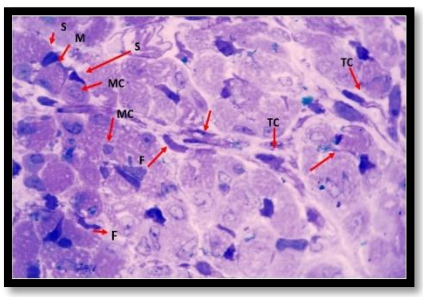


Figure 5: cross section showing myocyte (MC) matyrity by laying myofiliment with many other type of cells in the field : (F) fibroblast, (TC) telotyte, (S) satellite, (C) cell ,NDS, X 100

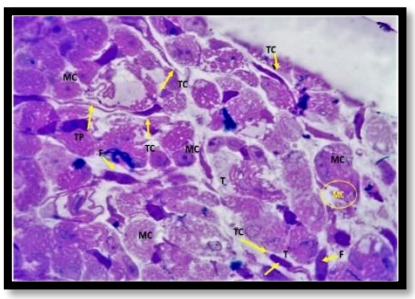
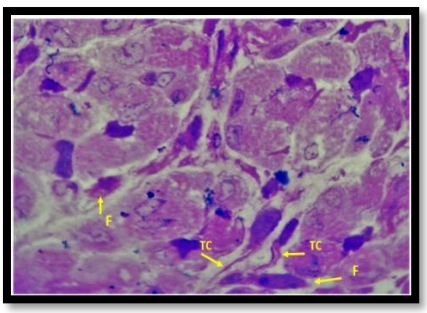


Figure 6: section showing gathering of myocyte (MC) & gaining maturity by myofibrile . (T) telocyte (TC) can be easly identified with specific feature of (TP) telopode, NDS. X 100



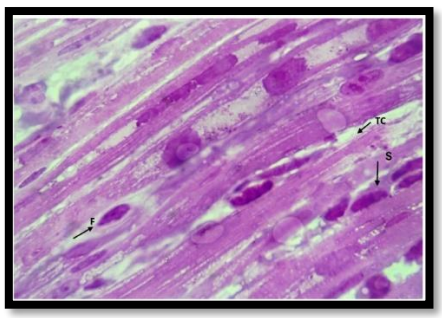


Figure 7: Section showing mature myofiber (M) & few telosyte (TC) can be seen between with adjacent fibroblast (F), NDS, X 100

Figure 8: Longitudinal section showing the arrangment of myofibers with periphery nuclei few are still central , telocyte (TC) , Fibroblast (F) , satellite sell (S) can be seen, ,NDS, X 40

Table 1 :- differences of p-value expression of CD34 the three groups.

Time period	Mean	Std. error	Minimum	Maximum	P1	P2	P3
First week	164738.67	24434.1	11571	418724	0.001	0.142	<0.001
Second week	290873.84	26813.4	28333	791305			
Third week	98389.8	16.895.5	26907	334599			

The mean value of positive pixle evaluated by Aporio Image scope Software for the third week group.

P1: between first and second week

P2: between first and third week

P3: between second and third week

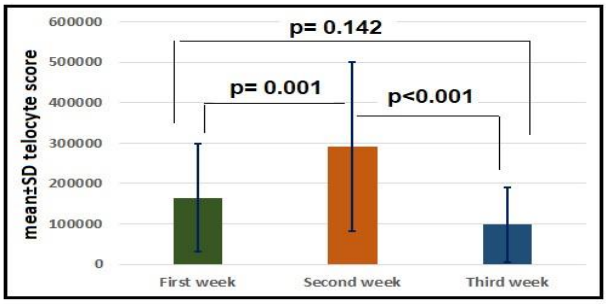


Figure 9: comparison of positivity between each pair of the three groups

Discussion

Assessment of different nuclei residence cells using NDS, Nuclei differentiated stain allow examine the cells closely and thoroughly being based very thin Sections (0.5Mμ) which give the stain this criteria. Area surrounding the bone were examined at first week of gestation showed many small size myoblasts in between mesenchymal connective tissue. Myoblast also known as (myogenic Progenitors cells of Mesoderm) are small cells with basophilic cytoplasm centrally located nuclei, the cells usually have irregular outline shapes, but its shape more rounded than other mesenchymal cells with abundant cytoplasm Mesenchymal cells are elongated cytoplasmic process, with the advancement of gestational age and around the beginning of the Second week of gestation, myoblast Started to gain increase the size and length.

NDS stain showed different types of cells that were different in their nuclear appearance, large cells with large centrally located nuclei were seen representing the growing myocytes, multiple other nuclei representing all type of connective tissue cells (fibroblast, mesenchyme cell, macrophages) and Satellite cell. One type of nucleus which was seen surrounding the periphery of growing myoblast with Its unique very long slim type of nucleus and very long cytoplasmic processes that extends away from the cells and seems to contact other cells which could be most probably represents telocyte.

According to previously published studies, TCs were identified by CD34 immunostaining using immunoperoxidase- based immunohistochemistry, CD34-expressing stromal cells were abundant, displayed a spindle-shaped appearance with long and moniliform processes, and formed a thin reticular network in the fetal skeletal muscle interstitium from all gestational ages. Therefore, these observations provided evidence that stromal cells with typical morphological features and immunophenotype of TCs were present in the human skeletal muscle during early myogenesis. Of note, the expression of CD34 in those stromal cells varied among the different gestational ages. Telocytes acquire cell prolongations or the telopodes that may reach hundreds of microns in length. They constitute a broad interstitial 3d network. Telopodes are composed of thin segments or podomers and dilated segment or podoms where mitochondria, endoplasmic reticulum are aggregated (Díaz-Flores et al., 2014; Manetti et al., 2015; Rosa et al., 2018).

Two pathways of cell communications have been mentioned for telocytes; cell contact and paracrine pathway. Various forms of cell contact are described between telocytes and other cells such as minute

junctions such as point contacts, nanocontacts and planar contacts and cell contact with intermembrane distance within which the macromolecules interact (Manetti et al., 2015). In this study only positive reaction were included in the assessment of CD34 antibody, reactivity in skeletal muscle, Statistical analysis (SPSS) showed CD34 reactivity in skeletal muscle this was assessed using Aperio Image Scope Software, the degree of positivity measured by this program ranged and expressed as follow: Immuno-histochemical reactivity CD34 Antibody showed an intense reactivity in 2 nd week group and a less intense reactivity in 3 rd week group.

Quantitative assessment of CD34 antibody in these three g roups showed statistically significant differences were P value ⩽ 0.001. Quantitative analysis of TCs and vessels was investigated by performed on fetal skeletal muscle sections double-immunolabeled with the mouse monoclonal anti-CD34 and the rabbit polyclonal anti-CD31/PECAM1 antibodies and counterstained with DAPI for nuclei. CD34-positive/ CD31negative TCs and CD34/CD31-double-positive vessels were counted in 10 representative microscopic high-power fields (hpf; ×40 original magnification) scattered in the preparation (i.e. 5 hpf with longitudinally sectioned myotubes and 5 hpf with transversely sectioned myotubes) per sample. Only the cells with well-defined nuclei were counted. Counting was performed by two independent observers who were blinded with regard to the sample classification. The final result was the mean of the two different observations for each sample (Mirca Marini, et al,.2018).

During the last few years, several studies have demonstrated the existence of a novel type of cell, namely TCs/CD34-positive stromal cells (formerly referred to as interstitial Cajal-like cells), in the interstitial space of different organs (Faussone Pellegrini and Popescu, 2011; Cretoiu and Popescu, 2014; Díaz-Flores et al., 2014; Cretoiu et al., 2017). In the adult skeletal muscle interstitium, TCs have been identified in close relationship with other cell types, such as satellite cells, striated muscle cells with regenerative features or even putative non-satellite progenitor cell niches (Bojin et al., 2011; Popescu et al., 2011; Marini et al., 2018). Moreover, the long cytoplasmic extensions of TCs (i.e. telopodes) form a three-dimensional interstitial network establishing multiple contacts with nerve endings, blood vessels and myocytes (Popescu et al., 2011). Hence, TCs have been proposed to play an essential role in intercellular signaling by either intercellular contacts or shedding of vesicles/exosomes with potential implications for skeletal muscle fiber regulation as well as muscle tissue development and post-natal regeneration/repair (Bojin et al., 2011; Popescu et al., 2011). Indeed, TCs might guide both satellite and non-satellite progenitor cells undergoing migration and differentiation after trauma (Popescu et al., 2011). On the basis of the expression of the stemness marker c -kit/CD117, some authors have also proposed that TCs could represent a unique progenitor cell type within human skeletal muscle stem cell niches (Bojin et al., 2011). It have been always postulated that TCs can only be followed by E.M, this study proved that TCs can been seen and followed using plastic section under light microscopy and using CD34 labelling. Rapid changes in CD34 expression and distribution of TCs in association with previously described morpho-functional skeletal muscle tissue modifications occurring during this critical period of organogenesis, including stromal organization, angiogenesis, apoptosis, cell interaction, contractility-relaxation and basement membrane formation (Marini et al., 2017).

According to substantial litterature (Pieri et al., 2008; Faussone Pellegrini and Popescu, 2011; Cretoiu and Popescu, 2014; Díaz-Flores et al.,

2014; Manetti et al., 2013, 2014, 2015; Alunno et al., 2015; Rusu et al., 2016; Rosa et al., 2018), CD34 marker to identify fetal skeletal muscle TCs under light microscopy. Moreover, we further carried out double immunofluorescence labeling for CD34 and the time window until 12 weeks of gestation in human fetal skeletal muscle. In fact, at 9 –9.5 weeks of gestation weakly CD34-immunolabeled TCs were detected in the stroma, while from 10 to 11.5 weeks of gestation these cells were increased in number and displayed a stronger CD34 immunoreactivity. it could be hypothesized that between 9 and 9.5 weeks TCs are presumably starting to undergo differentiation, which might be reflected by the acquisition of their characteristic CD34 antigenic profile, reaching the strongest immunoantigenicity between 10 and 11.5 weeks. This hypothesis is also supported by the evidence of a trend toward an increase in the number of fetal skeletal muscle TCs between 10 and 11.5 weeks of gestation.

Moreover, the decrease in the number and CD34 immuno -antigenicity of TCsthat we observed at 12 weeks mightsuggest that these cellssubsequently become quiescent and/or undergo degeneration, having presumably terminated their biological functions related to this myogenic phase. Interestingly, these changes in TCs appeared closely related to changes in amount and density of the stroma surrounding and inside myotube bundles, myotube behavior and number of blood vessels. Indeed, between 9 a nd 9.5 weeks, the stroma around and inside myotube bundles, consisting in closely grouped primary and secondary myotubes and myoblasts, appeared loose. Subsequently, from 10 to 10.5 weeks the stroma became dense and then returned loose from 11 to 11.5 weeks, when the primary and secondary myotubes were separating. Finally, at 12 weeks, the stroma surrounding bundles appeared again dense, quantitatively reduced and organized around small group of primary and secondary myotubes which were mostly separated and differentiating into mature myotubes. Interestingly, most of the studies on telocytes were done using adult age animal or human cases.

Therefore this study is designed to investigate existence, characteristics, and distribution of telocytes in skeletal muscle of rats embryo. It was also done to provide clues for better understand of their roles in normal and regenerative medicine using histological and immunohistochemical examination. Recently TCs are certainly discriminated from other interstitial cells (mostly fibroblasts) by their position, histological features and immunohistochemical characteristics.Fibroblast has short and thick cell processes that emerge from the cell body; it also has larger cell body with abundant rER. In addition, fibroblast has a different phenotype (c-kit negative) (Suciu, et al., 2010; Faussone-Pellegrini and Popescu, 2011).

Additional researches will be needed to expose the role of TCS in tissue development. In addition, further investigations are required to prove the possibility of TCs transplantation in manage ment of different pathological conditions.

Conclusion

Skeletal muscle formation during embryogenesis starts early in gestation were the dermomyotome were mainly the site where early myoblast was Identified.

The embryogenesis of Skeletal muscle is dynamic process started from myoblast, which form myocyte fuse together forming myotubes. which is turn with time showed the appearance of myofilaments.

Using a semithin Plastic Section (o.5 Micron) helped in identified telocytes in light microscopy Using especial Stain, the method Could open the door for a better understanding of Tcs in different tissue.

CD 34 Consider a reliable antibody for telocytes expression. being a Sort of progenitor cells. There was expression a significant varient in CD34 expression for telocyte in different gestational age. CD34 elevation during the 2nd of gestation Got could be attribute to the fact that the key role function of telocyte of cell to cell communication in probably highly demanded in the Period of gestation structural and function organization of muscle.

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