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Revisiting the Role of the Myofibroblast in Socket Surgery: An Immunohistochemical Study

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Purpose: To determine the impact of a single injection of various anti-inflammatory, antimitotic, and antiangiogenic agents on the cell count of myofibroblasts in an eviscerated socket.

Methods: One eye from 15 skeletally mature New Zealand white rabbits was eviscerated, and the rabbits were divided into 5 groups. Each group of 3 rabbits received a 0.1 ml subconjunctival injection of a different agent. Group I received bevacizumab 25 mg/ml, group II received triamcinolone 40 mg/ml, group III received 5-fluorouracil 50 mg/ml, group IV received mitomycin-C 0.4 mg/ml, while group V was the control group and received no injections. The animals were euthanized 19 days after evisceration and conjunctival samples were submitted for histopathological examination. Monoclonal α-smooth muscle actin antibody was applied, and the mean of 5 readings of the number of myofibroblasts was recorded in each slide.

Results: The mean count of myofibroblasts was highest for the control group and all groups achieved a statistically significant reduction in myofibroblast count compared with the control group. Sorting the means showed that Group IV (mitomycin-C) achieved the lowest mean value ($p = 0.000006$) followed by triamcinolone ($p = 0.00048$), while group I (bevacizumab) achieved the least reduction in myofibroblast count ($p = 0.00148$).

Conclusion: Until newer antimyofibroblast medications and antibodies are commercially available, a single injection of mitomycin-C or triamcinolone during surgery achieves the highest mean reduction of myofibroblast count.

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The myofibroblast, a major component in granulation tissue formation, is a uniquely differentiated fibroblast which has acquired smooth muscle characteristics, and consequently developed the exceptional ability to contract. This cell plays a transient yet detrimental role in generating the forces responsible for wound contraction, ultimately resulting in tissue remodeling and organ destruction. The scarring potential of myofibroblasts has been thoroughly documented in fibrotic situations throughout the body,[1–5] but is also implicated in other physiologic and pathologic processes including embryologic development and tumor pathogenesis.[2]

In response to a mechanical stress or challenge both in vivo and in vitro, some fibroblasts recruited at the site of injury first acquire contractile stress fibers composed of β- and γ-contractile actins forming an intermediary cell called the protomyofibroblast.[2,6] Only with the acquisition of α-smooth muscle actins (α-SMA) is the transformation from fibroblasts to myofibroblasts complete.[6] Thus, the presence of α-SMA is the most reliable marker for myofibroblasts.[2,6] Although this phenotypic transition of fibroblasts in myofibroblasts has been extensively studied in the literature,[1–5] the myofibroblast is not solely derived from fibroblasts and has multiple cellular origins,[2] including endothelial cells, smooth muscle cells, pericytes, epithelial cells, hepatic perisinusoidal cells, mesenchymal stem cells, and bone marrow-derived cells known as fibrocytes.[3]

While the pivotal role played by myofibroblasts in modulating wound healing, tissue remodeling, and organ deformation has received vast attention throughout the body, it has rarely been scrutinized in detail in the vicinity of the orbit, and despite the fact that it has been almost 30 years since the role of socket myofibroblasts in the pathogenesis of socket contraction was established,[7] only a single article further explored the possible role played by the myofibroblast in conjunctival wound healing.[8] Because in theory the myofibroblast could be an important target for wound healing modulation, and until the authors have a fuller understanding of the pathology of scarring, in this study the authors investigated the most effective commercially available wound modifying agent that could help keep these cells in check to reduce the contractile potential of the healing socket.

METHODS

An institutional review board approval was obtained for the study. All animals were treated in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Animal Ethics Committee at Ain Shams University approved and supervised the animal laboratory work included in this study. Fifteen male New Zealand rabbits were used in the study. The animals were anesthetized with a combination of a 5 mg/kg xylazine (xylaject, Adwia, Obour City, Egypt), and 30mg/kg to 50 mg/kg ketamine-HCL (Ketalar, Pfizer, New York, NY). Booster injections were given if required.

One eye of each rabbit was selected at random, and the surgical procedure was performed under sterile conditions with loupe magnification. In brief, 1 drop of 4% benoxinate oxybuprocaine (Epico, Cairo, Egypt) was applied topically to the eye before a 360° periotomy was performed, followed by excision of the corneal button, and then the intraocular contents were scooped. The operating surgeon was not masked to the type of injection given. The rabbits were divided into 5 groups. Each group of 3 rabbits received a single subconjunctival injection of a different agent in the same location near the superior fornix close to the vertical meridian in the midsuperior bulbar conjunctiva as follows:

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Group I: Received 0.1 ml of bevacizumab 25 mg/ml.
Group II: Received 0.1 ml of triamcinolone 40 mg/ml.
Group III: Received 0.1 ml of fluorouracil (5-FU) 50 mg/ml.
Group IV: Received 0.1 ml of mitomycin-C (MMC) 0.4 mg/ml.
Group V: Control group with no injection given.

The corneal and conjunctival wounds were not sutured, and an ophthalmic chloramphenicol antibiotic ointment was applied to the conjunctival sac. Postoperatively, the animals were provided with Tylenol solution mixed with their drinking water to alleviate the pain associated with evisceration. On day 19 after surgery, the rabbits were euthanized with an intracardiac pentobarbital sodium injection, after anesthesia was induced as already described. Every care was taken to obtain conjunctiva and Tenon capsule samples as close to the original injection site as possible using the center of the upper eyelid (12 o'clock position) as a guide. With a pediatric speculum in place to keep the conjunctiva taught, several drops of saline solution were applied to wet the conjunctiva but no fixative was applied before collecting the samples. Vannas scissors were used to cut a generous uniformly sized strip of tissue approximately 5 mm or 6 mm in diameter from all animals. The samples were immediately placed epithelial side upwards on a thin sheet of cardboard (surgical suture packaging) to flatten it and avoid wrinkling, and then the specimens with the cardboard were gently transferred to a vial containing freshly prepared fixative solution for later processing. The study pathologist attended sample collection and the acquired samples were quickly processed. For each case, serial sections were stained with haematoxylin and eosin (H&E).

Immunohistochemistry

Four micrometer sections of formalin-fixed and paraffin-embedded conjunctival samples were prepared. Immunohistochemical staining was performed using a primary clone 1A4 antihuman mouse monoclonal α-SMA antibody, prediluted to 1:50 dilution in 1% bovine serum albumin (Cat. #M0851, Dako, Carpinteria, CA, U.S.A.). Avidin–Biotin immunoperoxidase complex technique was used, by applying a super sensitive detection kit (Biogenex, CA, U.S.A.) according to the manufacturer instructions to detect the bound antibody. The prepared tissue sections were fixed on poly-L-lysine coated slides overnight at 37°C. They were deparaffinized and rehydrated through graded alcohol washes, then the sections were heated in a microwave oven in 10 mM citrate buffer (pH 6.0) for 10 minutes. After the blocking of endogenous peroxidase and incubation in Protein Block Serum-Free Solution (DakoCytomation, Glostrup, Denmark) for 20 minutes, the sections were incubated with α-SMA antibody at room temperature. Biotinylated antimouse immunoglobulin and streptavidin conjugated to horseradish peroxidase were then added, and then chromogen was used to form an insoluble brown product. Finally, the sections were counterstained with hematoxylin and mounted. Vascular smooth muscle was used as an internal control.

Quantification of Cells

In each specimen, the absolute number of labeled myofibroblasts in 5 randomly selected nonoverlapping, stromal fields were counted by the pathologist at ×400 magnification. This covered a horizontal area of 313.32 μm of the examined field, and a vertical area of 233.13 μm. To reduce possible investigator bias, this procedure was performed by the pathologist in a blinded fashion on serially numbered slides and the mean was used for the statistical analysis. α-SMA+ cells were counted using an Olympus microscope (Cx51) equipped with an Olympus camera using SIS software (Japan, Tokyo).

Statistical Analysis

The pathologist provided the data on an Excel spreadsheet (Excel 2010, Microsoft Corporation, Redmond, WA, U.S.A.), and statistical analysis was conducted with the SPSS software version 21 for Windows (IBM Corporation, New York, NY, U.S.A.). Statistical comparison was performed using the 2-sample *t* test allowing unequal variance. A *p* value less than 0.05 was considered statistically significant.

RESULTS

The results are summarized in Table. Qualitative examination of H&E sections showed an intact conjunctival surface epithelium in all groups, with the underlying stroma showing a dense chronic inflammatory cellular infiltrate in the control group. There was a reduction in the density of inflammatory cells in the other groups (Fig. A–C). Spindle shaped cells presumably myofibroblasts could be occasionally identified but were impossible to reliably quantify in H&E sections.

Quantification of myofibroblasts identified by α-SMA immunohistochemical staining showed that the mean count of myofibroblasts was highest for the control group. Sorting of the means showed that group IV (MMC) achieved the lowest mean value ($p = 0.000006$) followed by triamcinolone ($p = 0.00048$), while group I (bevacizumab) achieved the least reduction ($p = 0.00148$) in myofibroblast count (Fig. D–F), but all groups achieved a statistically significant reduction in myofibroblast count compared with the control group. Mitomycin-C also achieved significantly lower counts that bevacizumab ($p < 0.008$).

DISCUSSION

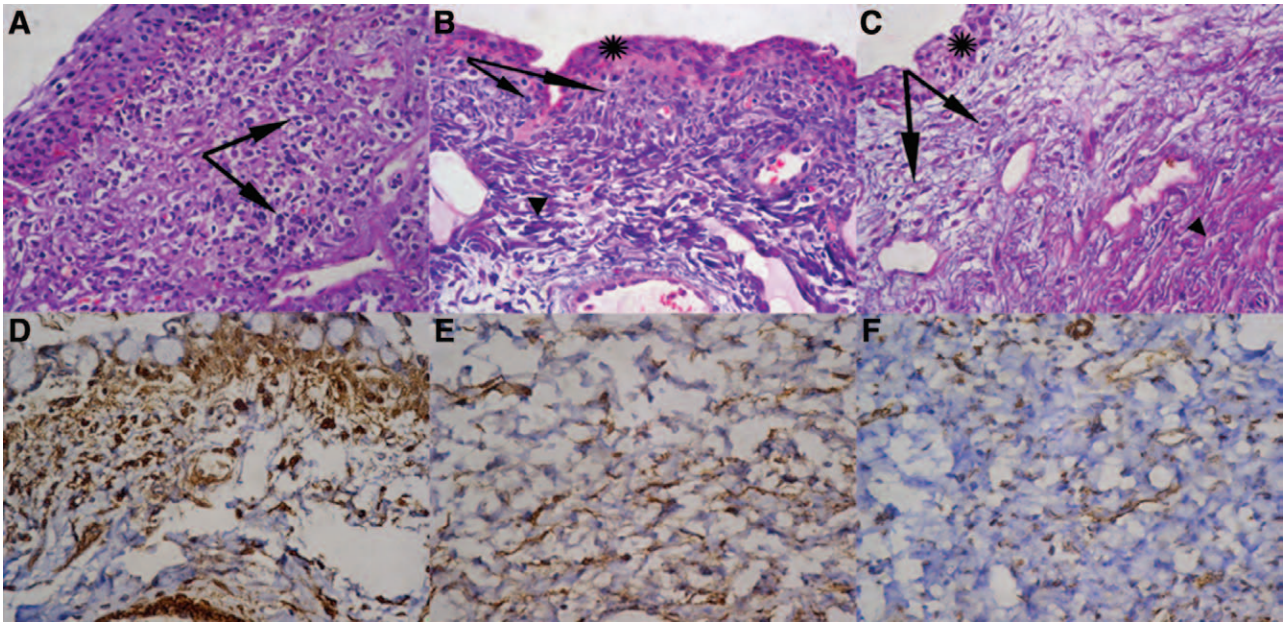
Because of the high failure rate associated with contracted socket surgery, oculoplastic surgeons have continually sought techniques to modify the conjunctival wound healing process.

Summary of the results showing that the highest mean count was in the control group (Group V) with all groups achieving a statistically significant reduction in myofibroblast count compared with the control group

	Group I Bevacizumab	Group II Triamcinolone	Group III 5-FU	Group IV MMC	Group V Control group
1st reading	252	204	254	212	291
2nd reading	271	230	225	239	311
3rd reading	249	210	264	210	337
4th reading	279	253	283	254	341
5th reading	276	266	245	215	326
Mean	265	233	254	226	321
SD	14	27	22	20	20
p value vs. group V (control group)	0.00148	0.00048	0.00102	0.000006	

The highest mean reduction was achieved in the MMC group (Group IV) followed by the triamcinolone group (Group II). Group IV (MMC) also achieved a statistically significant reduction of myofibroblasts compared with group I (bevacizumab; result not shown in the table).

5-FU, 5-fluorouracil; MMC, mitomycin-C; SD, standard deviation.



A–C, Sections in the conjunctiva (H&E, high magnification, ×400) showing an intact surface epithelium (*asterisk*). The underlying stroma showed a dense chronic inflammatory cellular infiltrate (*long arrows*) in the control group (**A**), and to a lesser extent in sockets injected with triamcinolone (**B**), and MMC (**C**), along with occasional myofibroblasts (*arrowheads*) that were impossible to quantify in H&E sections. **D–F**, α-SMA immunostaining of sections in the conjunctiva (high magnification, ×400, epithelium not shown) showing dense positive brown labeling of the myofibroblasts in the control group (**D**), with a progressive decline in the number of myofibroblasts in triamcinolone (**E**) and MMC sections (**F**). MMC, mitomycin-C; α-SMA, α-smooth muscle actin.

In 1987, Kaltreider et al.[7] were the first to demonstrate that the myofibroblast could be implicated as the cell responsible for contraction in the anophthalmic socket, but unfortunately since their original publication almost 30 years ago this harmful role has received little attention. It seems only sensible that the next step would have been to identify chemical agents that could help keep this cell in check, and although over the past several years the intraoperative or postoperative use of MMC or 5-FU as an adjunct drug during contracted socket surgery has shown some success in clinical studies;[9,10] to the best of the authors knowledge, no experimental studies corroborated these findings. Until newer medications that would help fight the action of myofibroblasts are commercially available, the authors sought to identify the most effective commercially available wound modifying agent that would help keep this unusual cell in check. Although the results of this study suggest that all the agents that were tried were effective in reducing the activity of myofibroblasts, the cell count decrement pattern was far from equivalent among tested agents, with MMC and triamcinolone achieving the highest mean reduction in myofibroblasts.

Because the authors did not report any toxic side effects from any of the agents used in the study, it could be easily argued that the adjunctive use of antimetabolites or anti-inflammatory agents in severe anophthalmic socket reconstruction is an effective option to control the scarring response, and could be effortlessly tolerated without side effects. The authors could also make unsubstantiated claims that this study lends further laboratory support to the aforementioned clinical studies[9,10] thus confirming the beneficial role of antimetabolites as an adjunct in contracted socket repair; however, this study has several inherent limitations that must be scrutinized in detail first.

First, it could be argued that it would have been more logical to apply these medications on an animal model for contracted socket because this is where the application of these agents will be put to actual use, however because the human body's fibrotic response is a universal response, building an animal model for socket contraction would have provided no additional value beyond the extensive mechanical trauma afforded with the generous periotomy and keratectomy performed in this study.

Another issue lies with the pathogenesis of socket contraction. A lot of contracted sockets are ischemic sockets with insufficient vascularization and it would seem counterintuitive to inject these sockets with wound modifiers that in essence would also lead to inhibition of new vessel formation where they are eagerly needed.[11,12] It should be argued, however, that although the chronically contracted sockets are certainly ischemic and even of lower temperature than regular sockets,[13] in actual clinical practice, the situation where these agents will be utilized is when a patient is operated on to repair socket contraction with mucous membrane grafting or other available grafting options. In this situation, when the conjunctival mucous membrane is breached in preparation for grafting, the socket becomes relatively "hot" postoperatively and a natural cascade is initiated where harmful new vessel formation is an integral part of the healing process with resultant recruitment of undesirable inflammatory cells that could potentially lay down scar tissue and convert to myofibroblasts which in theory would contribute to socket contraction.

Another possible weakness in this study is that 5-FU was given as a single injection, although traditionally glaucoma specialists recommend serial 5-FU injections to obtain its maximal effect[14] and indeed the inferior results obtained with 5-FU may possibly be attributed to inadequate dosing, yet recent studies have demonstrated the persistence of the intraocular pressure lowering effects of 5-FU for 6 full months after trabeculectomy augmented with a single intraoperative subconjunctival injection of 5-FU.[15] More importantly, myofibroblasts are transiently involved in the wound healing process in other parts of the body for 2 to 3 weeks only and therefore in theory any subsequent injections would be of no clinical value.[6] As the authors will discuss later, the most important advantage of 5-FU; despite its less than ideal inhibition of myofibroblasts compared with

MMC and triamcinolone, is that histological studies have demonstrated that unlike MMC, 5-FU has far less vascular endothelial[15] and probably also lesser epithelial toxicity.[16,17]

It is also surprising that bevacizumab worked. There are several mechanisms that could explain this dilemma. It seems that after tissue injury, fibroblasts express vascular endothelial growth factor in the extracellular matrix which functions as a pericyte and endothelial cell mitogen, a chemotactic agent, and an inducer of vascular permeability, which in effect would recruit more fibroblasts in the wound[18,19], a major physiologic source of tissue myofibroblasts. Another possibility is that pericytes in the newly formed blood vessels are myofibroblast progenitors themselves.[2]

Perhaps the major drawback in this study, and a major concern for the potential use of antiproliferative agents in contracted socket surgery is that although the authors demonstrated that the conjunctival epithelium generally remained intact after injections (Fig. B, C), they failed to quantitatively assess the impact of these agents on conjunctival epithelialization. The biosynthetic processes inhibited by antiproliferative or antimitotic agents do not only include subepithelial scar tissue formation but they may also impede or interfere with conjunctival cell replication,[17,20] which may negatively impact the ultimate outcome of contracted socket repair. Further research and/or clinical studies are warranted to figure out which agent at whatever particular concentration would pharamacokinetically strike the ideal balance between efficacy in inhibiting subepithelial scarring, and toxicity toward the epithelium.

In conclusion, the authors have demonstrated histologically that a single injection of MMC, triamcinolone, 5-FU, or even bevacizumab could significantly reduce the number of myofibroblasts in an actively healing socket. Until the emerging field of tissue engineering offers a viable conjunctival replacement, or until newer antimyofibroblastic medications become commercially available, a single injection of any of the aforementioned agents could theoretically reduce the contractile ability of the healing socket.

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