Histol Histopathol (2014) 29: 251-258
DOI: 10.14670/HH-29.251

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Detection of galectin-3 and localization of advanced glycation end products (AGE) in human chronic skin wounds

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Summary. The matricellular protein galectin-3 (Gal-3) is upregulated in excisional skin repair in rats where it has been shown to modulate the inflammatory phase of repair. Recent research into kidney pathology has implicated Gal-3 as a receptor for advanced glycation end products (AGE), resulting in the binding and clearance of these molecules. AGEs are thought to contribute to defective skin repair in diabetic patients as well as a result of the normal aging process. However, the distribution and localization of Gal-3 and AGEs has never been performed in human chronic skin wound tissue. Using immunohistochemistry, the localization of Gal-3 and AGEs in tissue isolated from chronic wounds and non-involved skin from the same patient was investigated. Of the 16 patients from which tissue was isolated, 13 had type II diabetes, one had type I diabetes and 2 patients without diabetes were also examined. In non-involved dermis, Gal-3 was detected strongly in the epidermis and in the vasculature. However, at the wound edge and in the wound bed, the level of Gal-3 labelling was greatly reduced in both the epidermis and vasculature. Labelling of serial sections for Gal-3 and AGE demonstrated that where Gal-3 immunoreactivity is reduced in the epidermis and vasculature, there is a concomitant increase in the level of AGE staining. Interestingly, similar labelling patterns were evident in diabetic and non-diabetic patients. The results from our study demonstrate an inverse correlation between Gal-3 and AGEs localization, suggesting that Gal-3 may protect against accumulation of AGEs in wound healing.

Key words: Advanced glycation end product, Chronic wounds, Diabetes, Galectin-3, Wound repair.

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Introduction

Diabetes Mellitus (DM) is a chronic disease which in 2010 affected more than 6.4% of Canadians over 12 years of age (James et al., 1998). Of these patients, a significant proportion of patients will develop an impaired wound healing response, which often results in development of chronic skin wounds or diabetic ulcers. For patients with DM, the lifetime risk of developing a chronic wound can be as high as 25% (Jaksa and Mahoney, 2010; O'Loughlin et al., 2010). Medical management of chronic skin wounds is often insufficient and lower extremity amputation (LEA) can be required. It is estimated that about 85% of LEAs are preceded by a chronic wound (Singh et al, 2005; Jaksa and Mahoney, 2010). Chronic wounds affect nearly 200,000 Canadians or 15% of the diabetic population, and with the prevalence of DM expected to rise significantly, new methods of treatment are desperately needed to reduce the morbidity and mortality associated with non-healing wounds (Elliott and Hamilton, 2011).

Immediately following an injury, platelets and the damaged vascular endothelium initiate the clotting cascade which creates a provisional hemostatic matrix

consisting of fibrin and platelets. Platelet degranulation is a source of clotting factors, PDGF, and transforming growth factor ß (TGFß) which are chemotactic for neutrophils (PMN) and macrophages (MP) that debride the wound (Mackie and Tucker, 1999; Midwood et al., 2004). In addition, the matricellular protein galectin-3 (Gal-3) is upregulated where it has been shown to assist in the recruitment of inflammatory cells (Liu and Rabinovich, 2010; Larsen et al., 2011).

The galectin family consists of 16 members which are part of a larger protein class known as the lectins that possess the unique ability to bind and transmit signals through their conserved carbohydrate recognition domain (Cao et al., 2002; Larsen et al., 2011; Konigshoff and Rojas, 2012). Gal-3, a 30 kD ß-galactosidase binding protein, is a unique matricellular protein in that it can be found within the nucleus, cytoplasm, cell membrane, the extracellular matrix, as well as serum (Liu and Rabinovich, 2010). The effects of Gal-3 are highly dependent upon its localization and the cell types with which it interacts. For example, intracellular localization of Gal-3 is protective against apoptosis of inflammatory cells which is in direct contrast to extracellular Gal-3 which has been shown to increase the rate of apoptosis of a variety of cells (Cao et al., 2002).

As well as modulating cell survival and apoptosis, Gal-3 has recently been implicated in the binding and clearance of advanced glycation end products (AGE) *in vivo* (Iacobini et al., 2004). The presence and accumulation of AGEs in the skin of diabetic patients is a well-documented phenomenon and has been correlated to increasing levels of diabetic vascular complications (Hu et al., 2012). AGEs, such as N-Carboxymethylysine are produced as a result of non-enzymatic glycation of carbohydrates and oxidized lipids to proteins. AGEs are not readily cleared from tissues and are also known to accumulate in the skin as a result of normal aging (Alikhani et al., 2005). Factors known to accelerate the deposition of AGEs in the skin include a slowed rate of protein turnover and prolonged hyperglycemia which are both present in patients with long-standing diabetes (Niu et al., 2008). AGE deposition can lead to the cross linking of extracellular matrix proteins which can result in alterations in signals regulating cell motility and adhesion.

As glycation of proteins is thought to impair wound healing, we examined human chronic wounds and surrounding tissue isolated from LEA. The aim of this study was to assess the extracellular matrix expression of Gal-3 in relation to AGE accumulation in human non-healing skin wounds.

Materials and methods

Tissue preparation

Human skin samples were obtained from patients undergoing elective lower limb amputations at London Health Sciences Center, Victoria Hospital. Patients with chronic non-healing dermal wounds were identified and consent was obtained. Three skin samples measuring $2cm^2$ were obtained from the edge of the lesion and an non-involved region of the limb. One sample from each region was then placed in 10% neutral buffered formalin to allow for transport from the hospital. Immunohistochemistry was carried out as described below. Chronic wound samples were obtained from 16 patients undergoing amputations at London Health Sciences Centre between 2009 and 2011 (Table 1). Samples from 13 male patients and 3 female patients were recovered with a median age of 68 years and an age range of 34 to 86 years. Of the 16 patients 13 patients were diagnosed with type two diabetes, one patient was diagnosed with type one diabetes, and two patients did not have

Table 1. Data collected from 16 patients who received a lower extremity amputation.

Sex	Age	Diabetic	Surgery	Remarks
Male	74	No	2009 Oct 21	Below knee, healthy from above ankle, wound from hell
Female	46	Type 2	2009 Oct 27	Below knee, healthy from above ankle, wound on lateral foot
Male	55	Type 2	2009 Nov 04	Above knee, healthy from above knee, wound at base of toes
Female	79	No	2009 Nov 17	Above knee but previous below knee, healthy from above knee, wound from site of previous amputation
Male	34	Type 1	2010 Jan 29	Below knee, healthy from above heel, wound on posterior heel
Male	70	Type 2	2010 Feb 05	Below knee, healthy from ankle, wound on lateral foot
Female	83	Type 2	2010 Mar 26	Below knee, healthy from ankle, wound on medial side of foot
Male	65	Type 2	2010 May 27	Above knee, healthy from below knee, wound on calf
Male	78	Type 2	2010 Jun 22	Foot amputated, healthy from ankle, wound on sole of foot
Male	66	Type 2	2010 Jul 15	Below knee, healthy from anterior ankle, wound on anterior foot
Male	56	Type 2	2010 Aug 13	Below knee, healthy from lateral foot, wound at site of previous toe amputation
Male	86	Type 2	2011 Jul 20	Below knee, healthy on dorsal foot, wound base of big toe
Male	58	Type 2	2011 Nov 08	Below knee, healthy from above ankle, wound side of heel
Male	47	Type 2	2011 Nov 08	Below knee, healthy from above ankle, wound anterior foot
Male	73	Type 2	2011 Nov 08	Below knee, healthy above ankle, wound lateral foot
Male	79	Type 2	2011 Nov 22	Below knee, healthy above ankle, wound anterolateral foot

Samples from 13 male patients and 3 female patients were recovered with a median age of 68 years and an age range of 34 to 86 years.

diabetes.

Immunohistochemistry and staining

Antibody dilutions were as follows: Gal-3 (SantaCruz) 1/200 and AGE (abcam) 1/10,000

Chronic wounds were excised and fixed in 10% neutral buffered formalin (Sigma Aldrich, St. Louis, Missouri). Paraffin embedded tissues were sectioned at 5 μm, mounted on positively charged glass slides and dried overnight at 42°C. Sections were cleared and rehydrated using standard methods. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 5 minutes. Sections were blocked with 10% horse serum and incubated with rabbit anti-AGE or mouse anti-Gal-3 (sc49480, Santa Cruz Biotechnology, Santa Cruz, California) primary antibody overnight at 4°C. Detection was by ImmPRESS Ig peroxidase kits (Vector Laboratories, Burlingame, California) and visualized with 3,3-diaminobenzidine (Vector Laboratories). Sections were counterstained with haematoxylin. Detection of Gal-3 or AGE was with appropriate primary antibodies (sc-32790, ab23722 respectively, Abcam plc, Cambridge, United Kingdom), where negative controls excluded the primary antibody. Trichrome staining was carried out as previously described (Lu et al., 2008).

Results

Trichrome Staining

Trichrome staining from the same sections illustrate

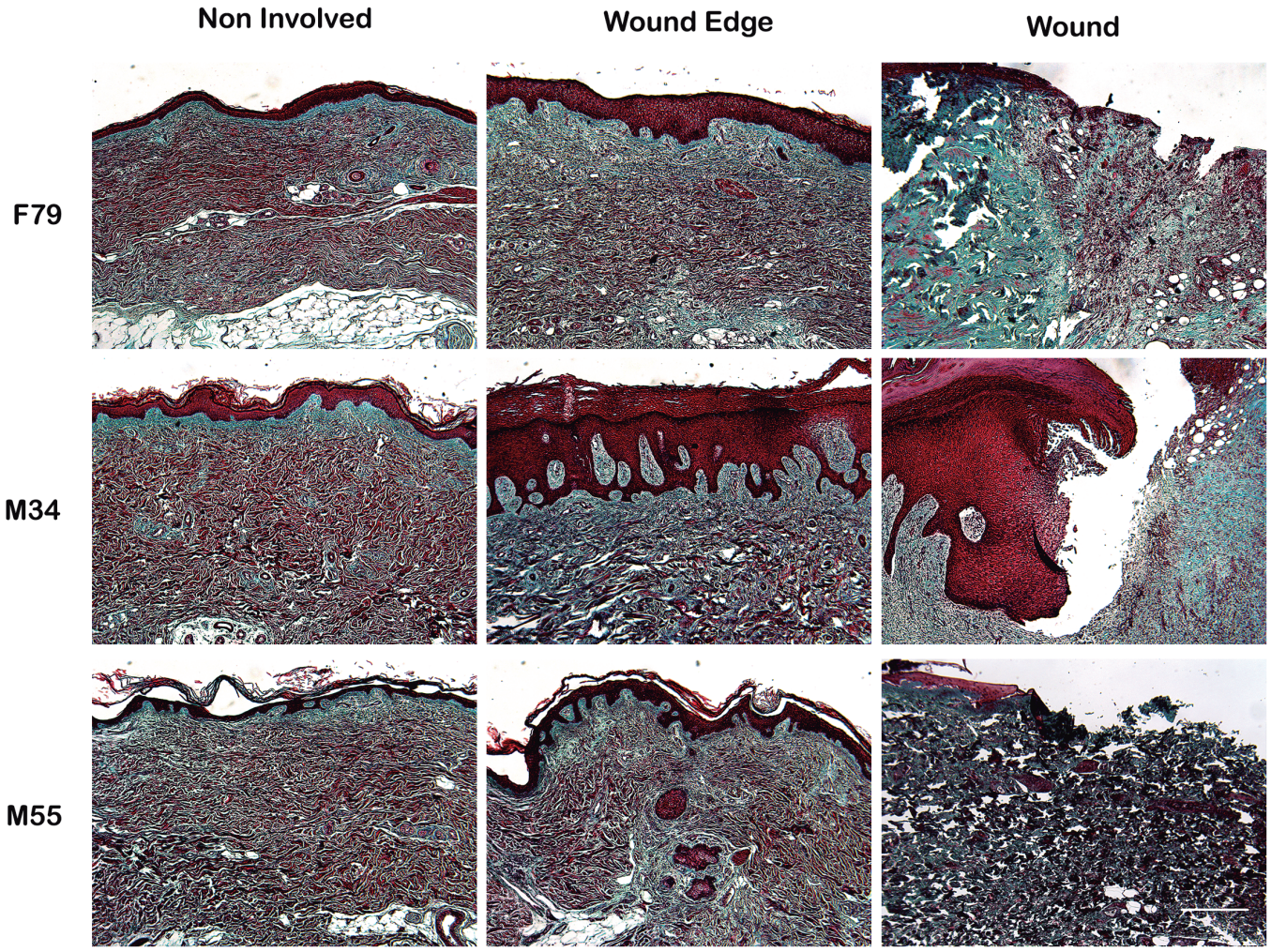

Fig. 1. Trichrome staining from a 79 year old non-diabetic female, a 34 year old type one diabetic male, and a 55 year old type two diabetic male. Sections from the non-involved tissue and wound edge reveal an intact epidermis stained (red) as well as strong collagen deposition in the dermis (blue). Within the wound there is a distinct loss of the epidermis with a large increase in collagen deposition corresponding to granulation tissue within the wound. Scale bar: 100 μm.

that in the non-involved tissue there is normal collagen organization in the dermal layer with a prevalent epidermis. At the wound edge and within the wound there is a dramatic reduction in collagen staining and a disorganized matrix corresponding to the deposition of granulation tissue and excessive inflammation in the wound bed (Fig. 1). Furthermore, the epidermal layer was disrupted at the wound edge and was typically completely missing within the wound site.

Galectin-3 staining

Immunohistochemistry staining for Gal-3 in all 16 patients demonstrated a difference in the localization of Gal-3 in the non-involved and wound region of the limb (Table 2). Staining for Gal-3 in the non-involved tissue reveals a strong band of staining restricted to the epidermis as well as staining in the vasculature (Fig. 2, Table 2). However, at the wound edge and within the wound the amount of Gal-3 staining is greatly reduced in both the epidermis and vasculature (Fig. 2, Table 2). In certain areas of the wound edge in certain patients, blood

Table 2. Qualitative quantification of Galectin-3 and AGE labelling of human chronic wound tissue.

Expression levels		Non-involved Skin	Wound Edge	Wound
Galectin-3	Epidermis	+++	+	-
	Dermis	-	-	-
	Vasculature	+++	-	-
AGE	Epidermis	-	+	++
	Dermis	-	+++	+++
	Vasculature	+	++	++

-, negative; +, mild; ++, moderate; +++, high staining intensity.

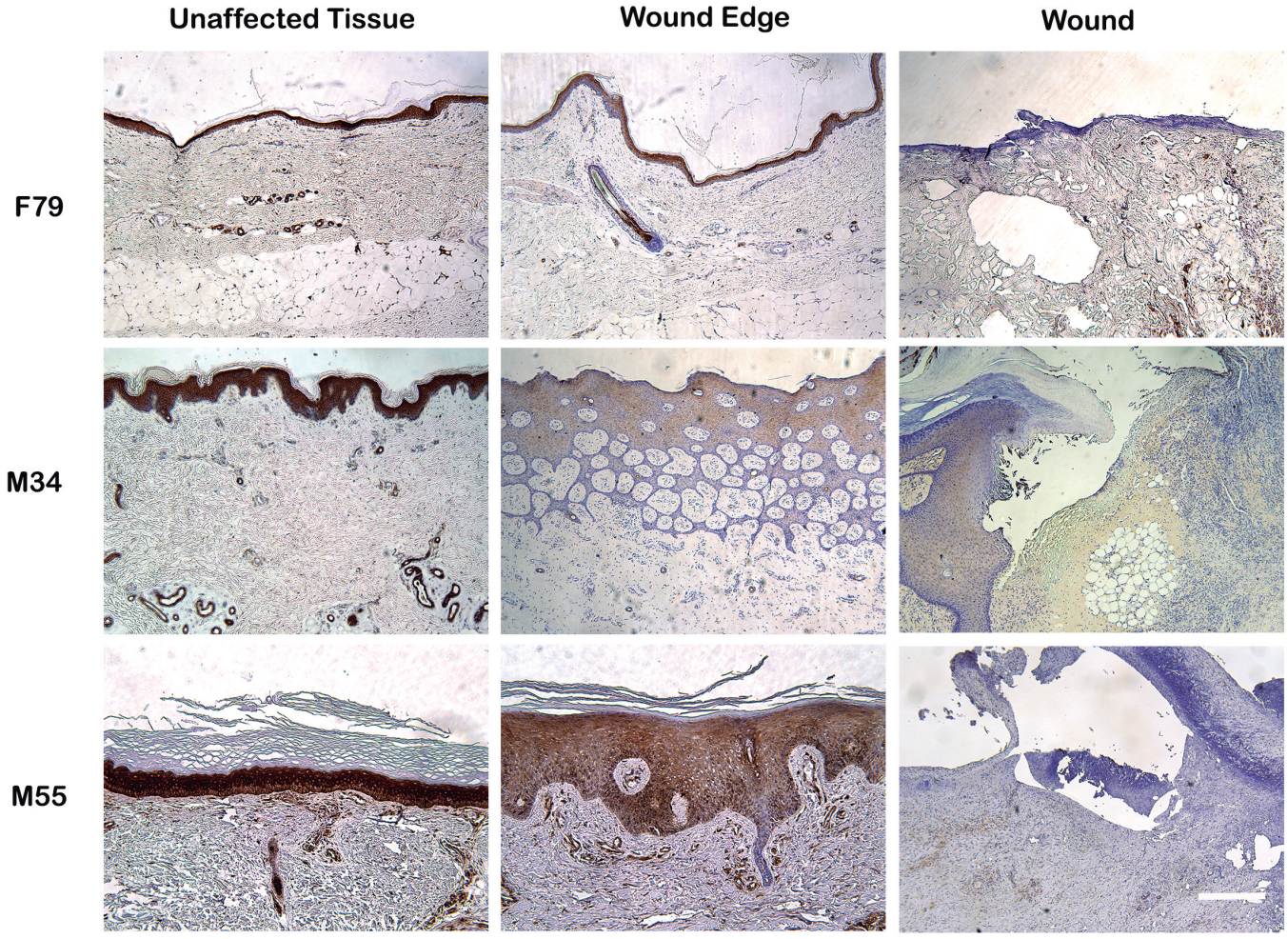

Fig. 2. Galectin-3 staining from a 34 year old male patient with type 1 diabetes, 79 year old non-diabetic female, and a 55 year old male patient with type 2 diabetes. Tissue from the non-involved region of the amputated limbs exhibit strong staining in the epidermis as well as the vasculature. In tissue more proximal to the wound bed Gal-3 expression is diminished slightly in the epidermis. In the wound bed Gal-3 staining is completely absent in the epidermis but still present in the vasculature. Scale bar: 200 μm.

vessels in the hypodermis stained strongly for Gal-3, but not blood vessels in the dermis (Fig. 3).

Galectin-3 and AGE staining

Serial sections from each patient were stained for Gal-3 and AGE. Results across all 16 patients demonstrate that AGE staining distal from the wound in the non-involved tissue is absent in the epidermis and vasculature but is present in the dermal layer (Figs. 3, 4, Table 2). At the wound edge and within the wound, staining for AGE increases further and is noted in the epidermis and the vasculature. Upon comparison of Gal-3 and AGE staining from the serial sections from the same patient, a pattern is observed in that as Gal-3 expression is reduced in the epidermis there is a concomitant increase in AGE staining in the epidermis (Figs. 3, 4, Table 2).

Discussion

In this study, we have demonstrated for the first time that the expression of Gal-3 in human chronic wounds is reduced in comparison with expression patterns evident in non-involved skin. Immunohistochemical studies demonstrate that Gal-3 is constitutively expressed within the epidermis and the vasculature in normal human skin. In support of this finding a study which investigated wound reepithelialization in rats demonstrated constitutive epidermal Gal-3 expression in non-wounded tissue (Gal et al., 2011).

Previous studies of wound healing in rats demonstrate Gal-3 is expressed in the first 3 days post-wounding where it helps recruit inflammatory cells to the wound to debride the wound bed (Rabinovich et al., 2002; Rubinstein et al., 2004; Saravanan et al., 2009). We demonstrate here that Gal-3 expression is reduced in the wound bed of chronic wound tissue compared with non-involved skin. Of great significance is that fact that although chronic skin wounds are stalled in an inflammatory state, Gal-3 expression does not persist. Therefore, this demonstrates that the expression of Gal-3 maybe important for initial inflammation (based on rat studies), but is not required in the wound bed for the

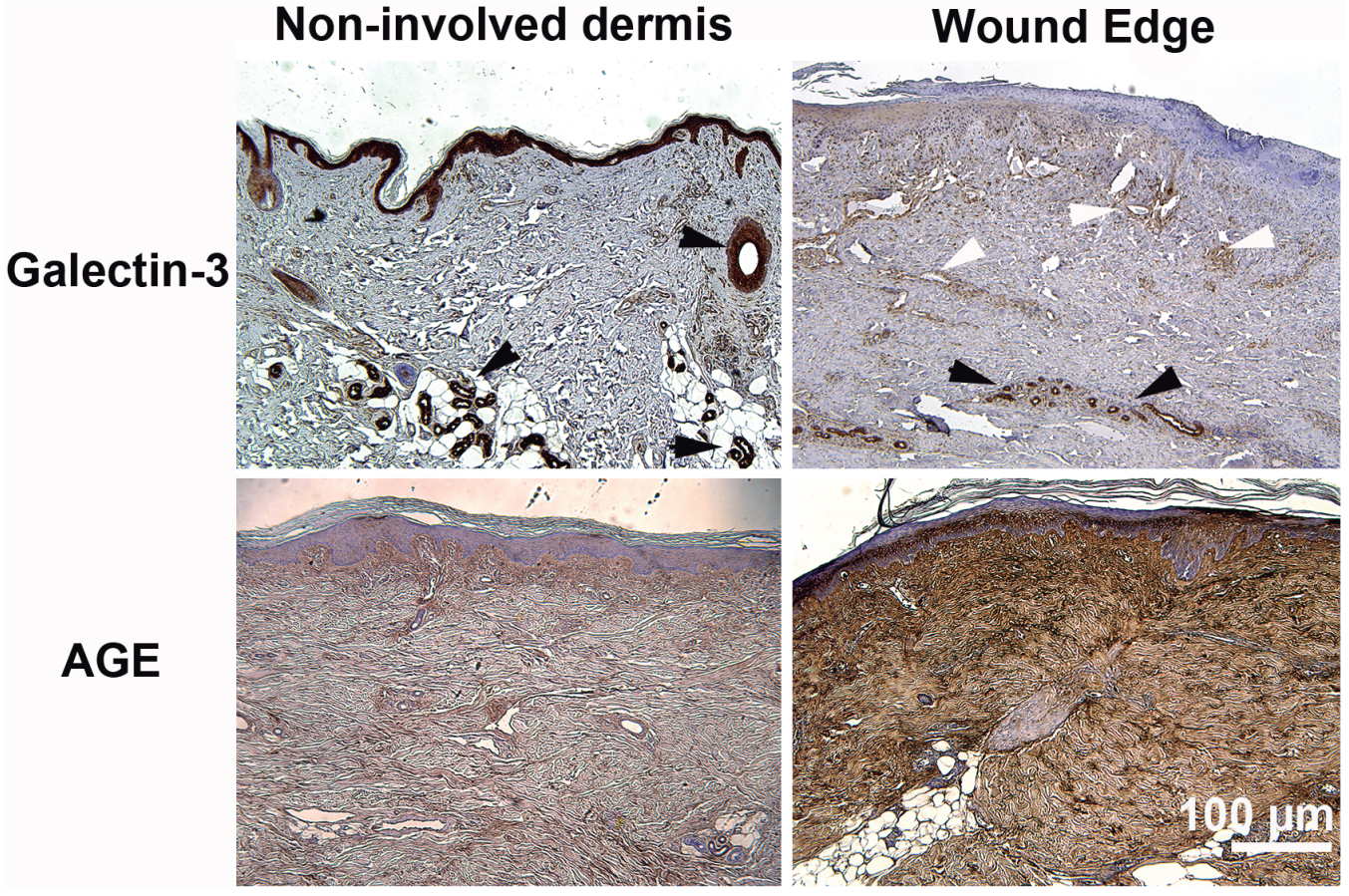

Fig. 3. Representative staining for Gal-3 and AGE compared between non-involved dermis and tissue at the wound edge. Gal-3 staining is strongly present in both the epidermis and vasculature distal from the wound but this decreases drastically at the wound edge. Staining for AGE reveals that in the non-involved tissue, AGE is not present in the epidermis but is present in the dermis. Towards the wound, AGE expression is increased throughout the dermis and a strong band of staining is also present in the epidermis. Black arrowheads highlight Gal-3 positive vasculature and white arrows vasculature with reduced Gal-3 immunoreactivity.

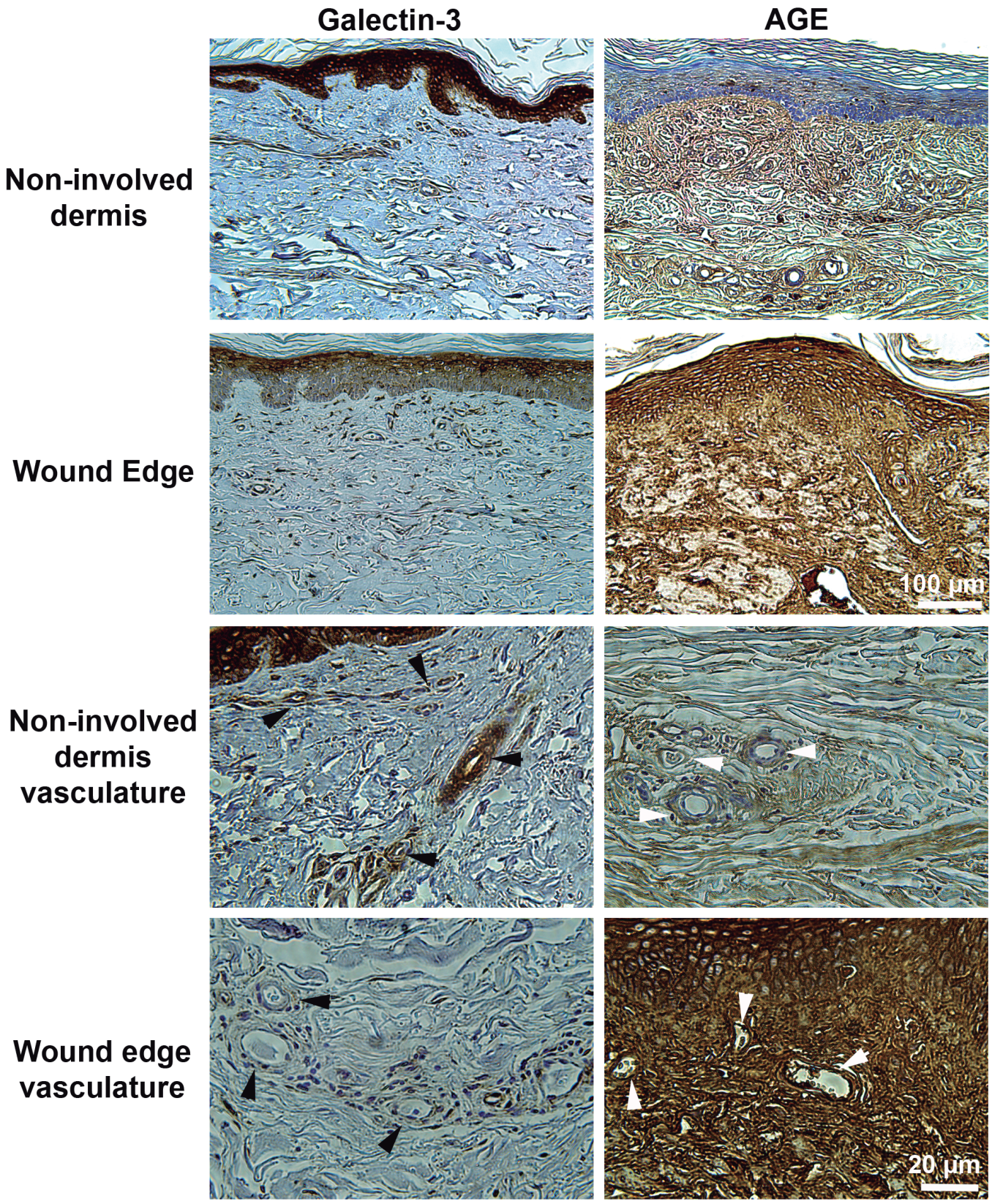

Fig. 4. Representative images of staining of the epidermis and vasculature for AGE and Gal-3 in a 55 year old male patient with type two diabetes. In the non-involved tissue there is strong staining for Gal-3 in the epidermis and vasculature and correspondingly AGE staining is prominent in the dermis and connective tissue surrounding the vasculature. At the wound edge there is a decrease in Gal-3 staining in the epidermis that corresponds to a band of AGE staining present in the epidermis. However, Gal-3 staining is prominent in the vasculature and as a result AGE staining is absent in the vasculature at the wound edge. Scale bar: 100 μm.

recruitment of inflammatory cells for wounds stalled in a chronic state. In contrast, our results demonstrate that in non-involved skin, where active tissue remodelling is absent, Gal-3 expression is present and is strongest in the epidermis (Fig. 3). Previous studies have also shown that Galectin-3 may results in damage to tissue after reperfusion injury in the kidney (Fernandes Bertocchi et al., 2008). Indeed, Galectin-3 is considered to be a marker of ischemic tissue and treatments aimed at reducing ischemia results in a decrease in expression of the protein in the brain (Dorai et al., 2011). Therefore, as we used tissue from lower extremity amputations, the possibility exists that Gal-3 expression could be higher in non-wound tissue due to increased levels of ischemia in these limbs. However, the chronic wound bed would be particularly ischemic and paradoxically Gal-3 expression is reduced. Future studies will focus on the relationship between ischemia in the limbs and level of Gal-3 expression.

As Gal-3 has also been implicated in the binding and removal of AGE, we next stained the tissue to assess the relative localization of glycated proteins surrounding the wounds. AGEs are a known ligand of Gal-3 and the absence of Gal-3 in KO mice lead to an exacerbation of AGE induced diabetic nephropathy (Iacobini et al., 2004; McFarlane et al., 2005). A clear understanding as to how AGE's accumulate or as to where they accumulate within the skin has yet to be investigated, particularly in relation to wound chronicity. Interestingly, when the expression of Gal-3 was compared to AGE it was observed that the Gal-3 expression corresponded to a reduction in AGE accumulation (Fig. 3). This relationship is most clearly noted in the vasculature (Fig. 4). Interestingly, a comparison of diabetic ulcers to ulcerative colitis reveals a similar pattern of Gal-3 expression in which it is expressed in non-involved tissue but not in the ulcer bed itself (Puthenedam et al., 2011).

In this study we were also able to comment on the effect of age and diabetes with regards to AGE accumulation. In particular examining the expression of AGE in younger diabetic patients requiring an amputation in comparison to older patients who also required an amputation there is a clear difference in the qualitative expression of AGE. Interestingly, despite this qualitative difference in the intensity of AGE staining both patients clinically still required an amputation. In the future, it will be important to study chronic wound formation in non-diabetic patients to assess the affect of aging alone on Gal-3 and in the histopathology of chronic wounds.

In future experiments it will be important to investigate other receptors which may contribute to chronic wound pathology, including other AGE receptors. Another important receptor to study is the receptor for advanced glycation end products or RAGE must also be considered in diabetic pathology. Previous reports have demonstrated that binding of RAGE to AGE signals through NF-κB to generate a pro-inflammatory response (Yeh et al., 2001). Target genes activated by this pathway include cytokines, adhesion molecules, and vasoconstrictive molecules, all of which play a major role in the normal wound healing response. CD36 is a class B scavenger receptor which is thought to play a central role in atherosclerosis and have recently thought to be involved in the progression of renal fibrogenesis. One study investigated chronic renal failure using CD 36 deficient mice noted that CD 36 deficient mice developed significantly less fibrosis in comparison to wildtype mice following ureteric obstruction (Okamura et al., 2009).

Overall, our results suggest that histologically the expression of Gal-3 and AGE do not overlap in chronic wounds. This pattern of expression needs to be further investigated *in vivo* and *in vitro* to assess if this relationship is of functional significance and whether manipulation of Gal-3 may aid closure of chronic wounds.

Acknowledgements. This work was funded by the Canadian Foundation for Innovation Leaders Opportunity Fund (18742), the Canadian Institutes of Health Research operating grants (MOP115044) to D. W. Hamilton, and the National Sciences and Engineering Research Council of Canada Canadian Graduate Scholarship program to C. G. Elliott. DWH is a recipient of the Ontario Ministry of Innovation Early Researcher Award.

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Accepted July 23, 2013