

Morphological Alterations of Microvasculature and Neoangiogenesis in the Pressure Ulcers Repair in Paraplegics

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Abstract

We performed a comparative study of histological, immunohistochemical and ultrastructural characteristics of blood microvasculature in specimens of pressure ulcers (PU) in paraplegic patients with acute spinal cord injury and in non paraplegic subjects. For immunohistochemical study we stained for cell membrane proteins that are commonly utilized for light microscopic characterization of blood endothelial cells as monoclonal antibodies CD31 and CD34, and for endothelial cell proliferation antigen Ki67. In paraplegics, the morphological and morphometric analysis of perilesional dermal tissues showed numerical capillary reduction, wall thickening and basement membrane changes of microvasculature. In non paraplegic ulcers the perilesional vasculature showed normal morphology. The granulation tissue in PU from paraplegics showed reduction in the number of new vessels and of the proliferating fraction of the endothelial cells if compared to non paraplegic PU. The onset of PU in paraplegics appears to be determined by mechanic factors and by intrinsic alterations of microvasculature such as impairment of angiogenic regulation in wound healing (VEGF and FGF2), and reduction of endothelial proliferation and capillary growth.

Key words: Spinal Cord Injury, Pressure Ulcer (PU), Microvascular Alterations, Immunohistochemistry.

Basic Appl Myol 15 (5&6): 203-208, 2005

Pressure Ulcers (PU) are a major complication after Spinal Cord Injury (SCI). The incidence of PU in SCI patients is about 30% and their pathogenesis is based on the hypoxemic hypothesis [18,26]. In the last decades, several functional and morphological studies on skeletal muscle of plegic limbs indicated that muscle fiber atrophy of the plegic muscle is associated with vascular bed changes. These involve capillaries, small venous, arterial vessels and lymphatics, and contribute to severe functional alterations in the microvasculature of disused skeletal muscle, as tissue hypoxia, increase in venous flow resistance and impairment of lymph drainage from subcutaneous tissues of the paretic leg [13,30-32]. These vascular changes are linked to thromboembolic disease, heterotopic ossifications, dystrophic skin alterations and pressure ulcers, commonly observed in SCI patients. They play important roles in their pathogenesis and history [18,21,29]. Some patients develop chronic PU in spite of the recommended medical therapy, rehabilitation treatment and correct nursing [3,4,11,22,32]. Numerous factors play a role in the ulcer healing pathogenesis and progress, as poor nutritional status, drugs and smoking, but the main pathogenic factor of PU is

hypoxia. The aim of the present study is to investigate with morphological, immunohistochemical and ultrastructural methods the microvasculature of the pressure ulcers from SCI patients in comparison to those of non-SCI patients.

Materials and Methods

Two groups of patients were studied. Group 1 consisted of 6 SCI patients (males, mean age 33 years, from 21 to 51) with IV grade PU requiring reconstructive plastic surgery located at sacrum, ischium or groin.

Group 2 (control group) consisted of 6 non-SCI patients with sacral or ischiatic PU, following prolonged post-surgical bed rest. These patients did not show pathologic alterations of microvasculature, such as diabetes or autoimmune diseases. After obtaining informed consent for this research, we sampled specimens from PU and from healthy perilesional tissues in SCI and non-SCI patients during plastic surgery in two Surgery Units of General Hospitals of Fiorenzuola and Pavia. Patients' characteristics are reported in Table 1. In Table 2 we have reported the characteristics of the PUs. Despite preventive measures (i.e. air pillow, air mat-

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Patient	Age	Sex	Lesion level	Asia Score	Ashwort Score	Years from lesion
D.S.	31	M	C4-C5	A	3	13
G.C.	21	M	C4-C5	A	3	2
J.M.	37	M	C5-C6	A	4	2
G.S.	35	M	T4-T5	A	3	1
N.G.	23	M	T8-T9	A	2	½
W.G.	51	M	T10-T11	A	2	2

Table 1. Patients' characteristics.

stress, mobilization exercises, daily skin check) and normal nutritional and clinical status (i.e., hemoglobin, platelets, haematocritous, iron), all of our patients developed PUs. All the PUs have been treated with a standard medical protocol: systemic and local antibiotic therapy, and specific aids to replace dermal stratum. Since this had been shown to be unsuccessful, the patients underwent osteotomy to level the bone under the wound and miocutaneous flap transposition.

	Ischiatic	Inguinal	Sacral
Evaluation PU Scale 4 th grade	2	1	3
Other pressure lesions	-	-	3
Precedent pressure lesions	2	-	-
Time between rising SCI and rising pres- sure lesions			
0-3 months	-	-	2
1-2 years	1	1	1
> 10 years	1	-	-

Table 2. Characteristics of the PUs

Morphology

Morphological, immunohistochemical and ultrastructural studies were performed on dermal capillaries, and on venous and arteriolar vessels from surgically removed specimens of PU at various levels: a) Specimens from the skin of border of the PU; b) Specimens from new granulative tissue at the margin of the PU; c) Specimens from the macroscopically normal skin and subcutaneous tissues in the peripheral areas of the lesion. Formalin-fixed and paraffine-embedded specimens were stained with ematoxylin and eosin, Gomori's trichrome stain and Periodic Acid/Schiff Stain (PAS)

Immunohistochemistry

Paraffine-embedded specimens were treated with the following mouse monoclonal antibodies versus: CD31 antigen (PECAM-1) that is a single chain membrane glycoprotein with a molecular weight of 140 kDa. The antigen strongly labels endothelial cells and it is expressed on the surface of blood cells and particularly at the endothelial intracellular junction [25]. Studies on the properties of CD31 antigen suggested that it is involved in events during angiogenesis and wound healing [10]. CD31 together with others immunoglobulin-related molecules, enable the phagocytes to squeeze between the endothelial cells and the basement membrane of vessels, and enables phagocytes to enter in the interstitial and subepithelial tissues for elimination of many pathogens. CD34 Class II antigen that is a single chain trans-membrane glycoprotein with a molecular weight of 110 kDa. The antigen is expressed on vascular endothelium, on connective fibres and basement membranes [20], and it is of value for the identification of vascular channels in granulation tissue of wound healing repair. Ki67 antigen is a large nuclear protein (345-395 kDa) expressed during all active phases of the cell cycle (G1, S, G2 and M phases), but absent in resting cells (G0 phase). In diagnostic histopathology, antibodies against Ki-67 antigen have proven valuable by allow indirect monitoring of the growth fraction of normal and neoplastic cells [33], and in the present study of the proliferating fraction of the endothelial cells. The specific binding sites of immunohistochemical stain for CD34, CD31 and Ki-67 are schematically indicated in Fig 1.

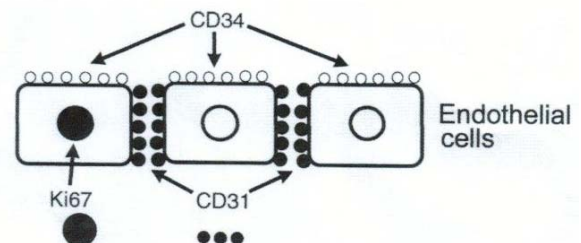


Figure 1. Immunochemical stains: specific binding sites of CD34, CD31, and Ki-67.

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Capillaries per Area Unit in granulation tissue of PU		Capillaries per Area Unit in perilesional normal tissue	
SCI-patients	Non SCI-patients	SCI-patients	Non SCI-patients
$20 \pm 2 / \text{mm}^2$	$56 \pm 4 / \text{mm}^2$	$68 \pm 6 / \text{mm}^2$	$88 \pm 8 / \text{mm}^2$
p=0,000001		p=0,000739	

Table 3 Morphometric results of the number of vascular channels and capillaries per square millimetre (capillary density) in the granulative tissue of pressure ulcers (PU) and in healthy perilesional dermal tissues.

Electron microscopy

Small specimens were fixed in glutaraldehyde-paraformaldehyde mixture in 0,1 sodium cacodylate buffer and then processed for light and electron microscopic analysis. Epoxy-resin embedded thin sections were stained with Giemsa and then observed under a light microscope. Ultra-thin sections were stained with uranyl acetate and lead citrate, and were observed with a Zeiss EM 109 electron microscope. Particular attention was paid to the analysis of endothelial cell and of intercellular junction morphology, of the basal membrane and pericyte structure and on perivascular connective tissue condition. Quantitative evaluation of the number and diameter of capillaries and small blood vessels was performed with an automatic Interactive Image Analysis System IBAS I and II (Kontron, Munich, Germany) on light and electron microscopy micrographs. Statistical analyses were performed by means of IBAS I system.

Results and Discussion

In Table 3 we have described the results of the morphometric analysis of capillaries and small vessels (that is, capillaries per Area Unit and capillary diameter) and the percentage of the proliferative Ki-67 positive endothelial cells. In comparison with non-SCI patients PU, in SCI-patients PU the histological and morphometric study of capillaries and small vessels from margins and bottom of the lesion demonstrated:

i) significant reduction in the number of capillaries per Area Unit; ii) increase of the mean capillary diameter together with thickening of vascular wall of the granulation tissues (Fig. 2 and 3).

The endothelial proliferation immunohistochemical marker Ki67 on capillaries and small vessels in SCI-patients PU showed a significant reduction of positive Ki67 cells in comparison with non SCI-patients PU. The immunohistochemical CD34 and CD31 antigens markers showed a reduced number of positive small vessels. These alterations indicate impairment in neoangiogenic processes in the granulative reparative tissue and indicating alterations of the endothelial cell adhesion and of phagocyte diapedesis through the vessel wall in SCI-patients PU. The morphometric study of perilesional skin tissues from PU showed significant reduction in the capillary and small blood vessel number in the dermis of SCI patients in comparison with non-SCI patients (Fig 4 and 5). In SCI patients PU, morphological and

ultrastructural studies showed reduplication and thickening in the basal lamina of small vessels (Fig.2) and diffuse enlargement and dilatation of venous vessels with congestion of arterioles and capillaries. Similar alterations were not seen in non SCI-patients perilesional tissues.

SCI patients are particularly at risk of developing PUs. The physiopathology of the PU evolution is widely known as the result of an ischemia derived from an important pressure on capillaries. This process is justified by a physical cause [7]. In spite of medical treatment, PU in SCI-patients progresses in a chronic fashion and the healing processes are impaired. Numerous pathogenetic factors, as poor nutritional status, drugs and im-

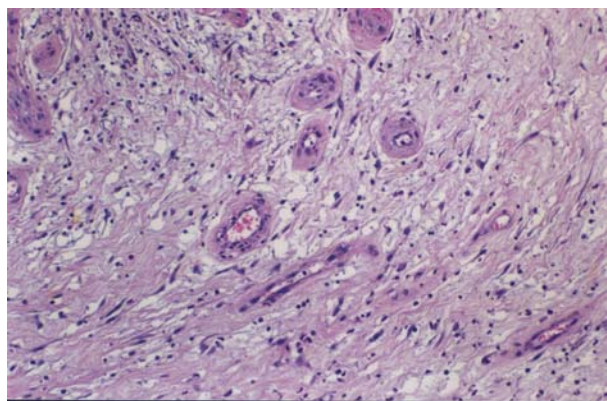


Figure 2. Wall thickening of dermal capillaries in perilesional tissue from a paraplegic patient with pressure ulcer. H and E. (250X).

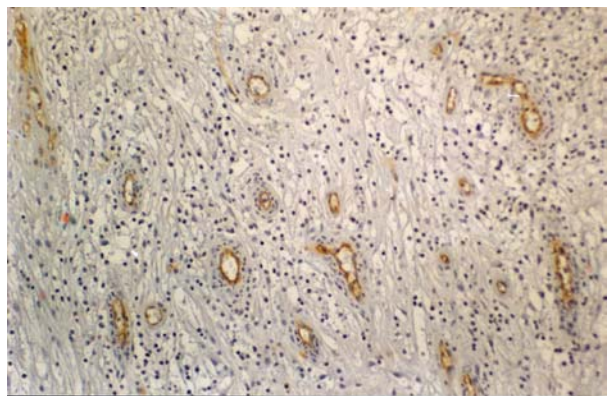


Figure 3. Neoangiogenic vascular component in the granulative tissue from a pressure ulcer in a non paraplegic patient. Staining with CD31 (150X)

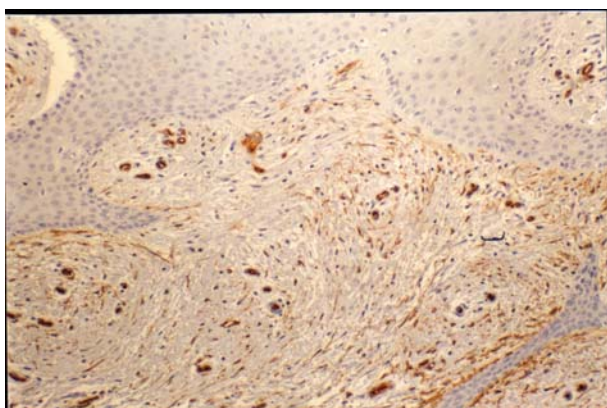


Figure 4. Some microvascular channels surrounded by rare and dissociated connective fibrils in the granulation tissue of pressure ulcer from a paraplegic patient. Staining with CD34 (100X)

munofunctional alterations were considered [6], but the pathophysiology of PU development is largely acknowledged to result to ischemia by exceeding tissue capillary pressure i.e capillary close pressure [19]. In the wound healing of non SCI-patients, angiogenic processes become active 2 days after the onset of PU and the severe degree of hypoxia in granulation tissue most likely results from both disruption of the native vasculature and increase oxygen consumption by cells in the wound environment. During angiogenesis, endothelial sprouts derive from intact existing capillaries at the wound normal peripheral tissue [9,14] and the angiogenic processes are regulated by a variety of cytokines as vascular endothelial growth factors (VEGF and FGF-2) [5,12,24]. Investigating the immunophenotype of proliferating vessels and of neoformed vascular channels in granulation tissues from skin ulcers, there was found identical immunophenotype features in normal human skin [28]. By using specific markers as CD34, CD31 and Ki67, we have observed initial repair, neoangiogenesis and slowed cicatrization processes. Several physiological and morphological studies have demon-

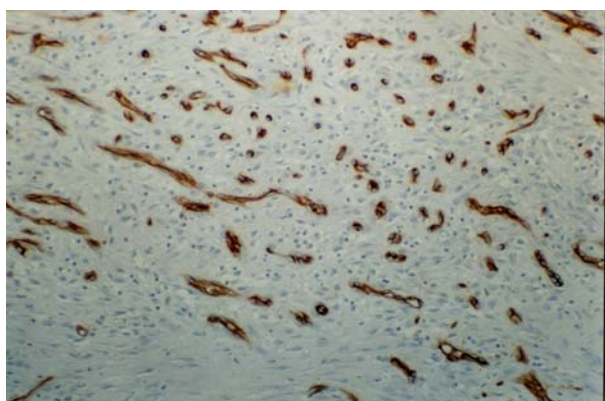


Figure 5. Numerous neoformed vascular channels in the granulation tissue of pressure ulcer from a non paraplegic patient. Staining with CD34. (150X).

strated important changes of the microvasculature of the paretic legs after SCI, with reduction of perfusion pressure and enhancement of the leg vascular resistance. In comparison to normal capillary bed, SCI-patients at the first stage of PU, showed a significant reduction of the number of capillaries and enlargement of small venous and arterial vessels in the skeletal muscle of the paretic leg. In long-term SCI, the reduction of the capillarity was associated with anatomical changes of the microvasculature, as vessel wall thickening and reduplication of the basal lamina [31]. These changes in the paretic muscle were associated to similar morphological alterations in the microvascular bed of the skin, involving both blood and lymphatic vessels [29]. The present study focused on the comparison of morphological, immunohistochemical and ultrastructural aspects of microvasculature in PUs in SCI and non-SCI patients. Our results indicate that the capillary growth and endothelial cell proliferation are impaired in the granulation tissue produced for the repair of PUs in SCI-patients. It is possible that the SCI-patients microangiopathy in association to loss of the vascular sympathetic control, and to deregulation of vascular cytokines and of some glycoproteic antigens (CD34 and CD31) involved in angiogenesis and wound healing, may play an important role in the development of the granulation tissue and angiogenesis in SCI PU. Recently Authors demonstrated that the neoangiogenic regulation in the wound repair by VEGF and FGF-2 is impaired in denervated muscle and that the intermittent endurance training has some effect on endothelial proliferation and on distribution of the vascular growth factors in the human skeletal muscle [15].

These results suggest that to improve the healing of wound is necessary a specific stimulation which could induce the production of VEGF or, in substitution the PU can be treated by topical treatment with cytokines and growth factors. Production of growth factors is stimulated by movements and the physical exercise [27]. Physical therapy [4], walking with aids [8,13] or with Lokomat or Treadmill [1,34] and correct use of FES [2,16,17] can be particularly important in the prevention of PU. An other important role is represented by the education of SCI patient to prevent PU with automobilization of paretic limbs, with specific draining postures to prevent venous blood stagnation and the tissue anoxia, with a correct diet to prevent thickening of vessel wall. We also observed that in spite of similar life-style, preventing measures and physical treatment, only some SCI-patients developed PU, thus supporting the hypothesis of a genetic predisposition due to apoptosis processes [35,36].

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