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Research paper

Determination of skin wound age by using cytokines as potential markers

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ABSTRACT

Interleukin-1 beta (IL-1β), IL-6, tumour necrosis factor-alpha (TNF-α) and epidermal growth factor (EGF) play important roles in the wound healing process. In the present study, human wound specimens (n = 50) were collected from cases of death due to injuries from firearms, penetrating trauma by sharp objects and blunt trauma with a known time of injury and death identified by forensic autopsy. Full-thickness tissue specimens were obtained from injured skin sites, and equally sized intact tissues obtained from the same person were used as controls. Protein determination was performed using ELISA according to the Bradford method for each specimen, and results were provided for individual proteins. IL-1β levels did not reach statistical significance in any of the wound groups and were not markedly higher than those in the control group. However, IL-6 showed a biphasic pattern and reached statistical significance in the group with wounds less than 30 min old and in the group with wounds more than 18 h old. IL-6 was consistently higher in all wound groups than in the control group. TNF-α showed a statistically significant increase within the first 30 min and remained at a high level in all groups except for those with wounds 2–4 h old. On the other hand, EGF was high in all groups excluding those with wounds 2–4 h old and more than 18 h old, but statistical significance was not reached. Our results suggest that IL-6 and TNF-α in particular may be used as early-phase markers. We believe that IL-1β and EGF should be more extensively evaluated in further studies.

1. Introduction

Determining the exact duration from injury until death by wound age estimation is of utmost importance in medico-legal death cases. The most common question in daily forensic medicine practice is related to the cause of death, followed by the questions 'How long did it take before the person in question died?' and 'How long could the person have survived with this wound?'.[1,2] Determination of wound vitality and age has been associated with several challenges in autopsy cases. Wound age determination has an important place in the field of forensic medicine for answering the above questions. Estimating wound vitality and age is crucial for the proper course of legal proceedings.[1,3] Several scientific studies have been reported using different techniques that help to resolve these problems.[4–13]

A wound is defined as an impairment or loss of integrity of different dermal or mucosal structures owing to a number of reasons, which lead to temporary or complete loss of their inherent physiological characteristics. Wound healing is a vital process that occurs in response to tissue injury and can be defined as a series of sequential and interrelated cellular, physiological and biochemical events. Wound healing is still a major area of interest for scientists owing to the fact that its underlying mechanism has not been fully understood.[14,15]

The wound healing process begins within the first few minutes after an injury[1,16,17] and may last for days, months and even years.[1] Wound healing comprises three separate, interconnected phases: a) haemostasis and inflammation, b) proliferation and c)

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maturation and remodelling.[1,18,19] Following trauma, platelet aggregation is initiated when platelets released from injured blood vessels come into contact with subendothelial collagen, which sets off the coagulation mechanism. The platelet coagulation resulting from this contact and thrombin and fibronectin that are already present in the environment lead to the release of cytokines from alpha granules of platelets.[1,19] Cytokines are cellular regulatory proteins released from specialised cells in response to various stimuli and affect the behaviour of target cells. Cytokines include growth factors, lymphokines, colony-stimulating factors, transforming growth factors, tumour necrosis factors (TNFs) and interferons.[20] Cytokines are markers that exhibit a surge particularly in the early stage of wound healing.[21]

Histochemical studies have been conducted in this area over the last 25 years.[22] Recently, immunohistochemical methods have mostly become the focus of studies on wound healing. Injuries of the skin and certain other organs have been studied on the basis of experiments on animals and investigations of emergency room, surgical and autopsy cases. Far fewer relevant studies have been performed on autopsy cases than those on other types of cases.[23–28]

Certain conditions affect the timing of release and amount of cytokines. Among these, severe malnutrition, malignant diseases and metabolic disorders, medical therapies with glucocorticoids and cytostatic agents and exposure to chronic radiation have several unfavourable effects on wound healing and delay the healing process; differences in cytokine values have been demonstrated at such instances.[19,29] Cases associated with such conditions were excluded from the present study.

Cytokines are polypeptide molecules produced by a broad range of cells, including activated lymphocytes and macrophages; they are actively involved in the regulation of the functions of other cells.[30,31] Cytokines can act on the cells that produce them as well as other cells in the close vicinity and have systemic effects. Cytokines have local effects on the endothelium, leukocytes and fibroblasts.[31]

Cytokines modulate various biological events and play important roles in the immune, central nervous, endocrine and haematopoietic systems. The interleukins (ILs) IL-1 and IL-6 as well as TNF-α are pro-inflammatory cytokines that are constitutively present in keratinocytes and the sweat glands of uninjured skin.[32]

Disruption of the epidermal barrier results in the release of the pre-stored IL-1 and TNF-alpha (TNF-α) from keratinocytes. IL-1 and TNF-α alert the surrounding cells regarding signs of barrier damage. In addition, tissue injury causes blood vessel disruption with concomitant extravasation of blood constituents. The resulting clot induces haemostasis and provides a matrix for the influx of inflammatory cells. Platelets also secrete growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) and several chemokines. These proteins promote the recruitment of inflammatory leukocytes to the site of injury. Infiltrating neutrophils remove contaminating bacteria and then are extruded with the eschar or phagocytosed by macrophages. Subsequently, monocytes are differentiated into macrophages and recruited at the injured sites. Macrophages play a crucial role in augmenting the inflammatory response and tissue debridement. Macrophages also initiate the development of granulation tissue and release various proinflammatory cytokines (IL-1 and IL-6) and growth factors [fibroblast growth factor (FGF), EGF, TGF-β and PDGF]. Within hours of injury, re-epithelialisation is initiated and the release of EGF, TGF-α and FGF acts to stimulate epithelial cell migration and proliferation. This process begins with the dissolution of cell–cell and cell–substratum contacts, followed by the migration of keratinocytes over the provisional extracellular matrix (ECM).[33]

In the current study, IL-1β, IL-6, TNF-α and EGF markers were studied using ELISA in specimens of human skin wounds obtained at autopsy. The results were compared with those of normal skin specimens to assess their utility for the determination of skin wound age.

2. Material and methods

Cases of death following injuries from firearms, penetrating trauma by sharp objects and blunt trauma with a known time of injury and death identified by forensic autopsy between 25.02.2010 and 03.10.2010 at the Morgue Specialization Department of the Council of Forensic Medicine, Trabzon Branch, Turkey, were included in this study. Subjects from 10 to 80 years of age were evaluated and those with severe malnutrition, malignant diseases and metabolic disorders, undergoing medical therapies with glucocorticoids and cytostatic agents and exposed to chronic radiation that could interfere with the study assessments were excluded. Specimens obtained from wound sites and intact skin tissues were divided into five groups based on the time until death: those who died within 30 min, from 0.5 to 1 h, from 2 to 4 h, from 6 to 12 h, and 18 h or more after injury. This classification was arbitrarily chosen based on the time intervals within which the subjects died. Full-thickness tissue specimens of injured skin sites (0.5–1 cm in length and 0.5 cm in width) obtained from the bodies and an intact skin tissue specimen of equal size obtained from the same person as a control were stored at −80 °C until the time of analysis.

2.1. Biochemical assessment

Neat, fat-free sections weighing 50–100 mg were obtained from frozen tissues on ice and homogenized in PBS buffer, pH 7.4, containing 2 mM phenylmethanesulfonyl chloride, 5 mM ethylenediaminetetraacetic acid and 1 μg/mL each of leupeptin, antipain, aprotinin and pepstatin A protease inhibitors using an Ultra-Turrax T-25 model homogenizer. Following homogenization, sections were sonicated for 30 s using a Sonics sonicator and then allowed to agitate for 1 h at 4 °C for extraction and centrifuged for 20 min at 15,000*g* and 4 °C with a Beckman Coulter centrifuge. EGF (Ray Bio, Cat: ELH-EGF-001), IL-1β (Dia source lot: 101804), TNF-α (Dia source lot: 100801/B) and IL-6 (Dia source lot: 100402/B) were obtained in the supernatant using an ELISA kit. Supernatant was diluted 5- to 10-fold and protein determination was performed according to the Bradford method for each specimen. Results were obtained for individual proteins.

2.2. Statistical analysis

The assumption of normality of the data was checked using the Shapiro–Wilk test. To compare wounded skin versus normal skin, the Wilcoxon's rank sum test was used, which is suitable for non-normal data. Median and interquartile range (25%–75%) were provided as descriptive statistics. All analyses were performed using SPSS for Windows version 22.0. A two-sided P value of less than 0.05 was considered statistically significant.

3. Results

Out of the 50 cases enrolled in the study, 22 cases were of firearm injuries, 10 cases of sharp object injury, nine cases of falls from a height, six cases of road accidents, one case of assault, one case of injury from a falling tree and one case of crushing–penetrating injury. The mean age of the subjects was 41.12 ± 18.23 years (range 10–80 years), and 44 of the subjects were male (88%).

IL-1β values were comparable with those in the control group