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**ANA 404: INTRODUCTION TO HISTOPATHOLOGY**

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**QUESTION 1: Write on cytokine signaling and its role in wound healing.**

Cytokines are small secreted proteins released by cells have a specific effect on the interactions and communications between cells. Cytokine is a general name; other names include lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocyte and acting on other leukocytes) (Zhang & An, 2007). Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action) (Zhang & An, 2007).

Cytokines are made by many cell populations, but the predominant producers are helper T cells (Th) and macrophages. Cytokines may be produced in and by peripheral nerve tissue during physiological and pathological processes by resident and recruited macrophages, mast cells, endothelial cells, and Schwann cells. Following a peripheral nerve injury, macrophages and Schwann cells that gather around the injured site of the nerve secrete cytokines and specific growth factors required for nerve regeneration (Zhang & An, 2007). Localized inflammatory irritation of the dorsal root ganglion (DRG) not only increases pro-inflammatory cytokines but also decreases anti-inflammatory cytokines (Zhang & An, 2007). Cytokines can also be synthesized and released from the herniated nucleus pulposus, synthesized inside the spinal cord, the DRG soma, or the inflamed skin. Furthermore, cytokines may be transported in a retrograde fashion from the periphery, via axonal or non-axonal mechanisms, to the DRG and dorsal horn, where they can have profound effects on neuronal activity and therefore contribute to the etiology of various pathological pain states (Zhang & An, 2007).

Proinflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. There is abundant evidence that certain pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are involved in the process of pathological pain. IL-1β is released primarily by monocytes and macrophages as well as by nonimmune cells, such as fibroblasts and endothelial cells, during cell injury, infection, invasion, and inflammation (Zhang & An, 2007). Very recently, it was found that IL-1β is expressed in nociceptive DRG neurons. IL-1β expression is enhanced following crush injury to peripheral nerve and after trauma in microglia and astrocytes in the central nervous system (CNS). IL-1β can produce hyperalgesia following either intraperitoneal, intracerebroventricular or intraplantar injection (Zhang & An, 2007). Moreover, IL-1β was found to increase the production of substance P and prostaglandin E2 (PGE2) in a number of neuronal and glial cells. IL-1ra, a specific IL-1 receptor antagonist, competitively binds to the same receptor as IL-1β but does not transduce a cellular signal, thereby blocking IL-1β-mediated cellular changes (Zhang & An, 2007). Administrations of IL-1ra and other anti-inflammatory cytokines have been demonstrated to prevent or attenuate cytokinemediated inflammatory hyperalgesia and nerve-injury induced mechanical allodynia (Zhang & An, 2007).

IL-6 has been shown to play a central role in the neuronal reaction to nerve injury. Suppression of IL-6R by in vivoapplication of anti-IL-6R antibodies led to reduced regenerative effects (Zhang & An, 2007). IL-6 is also involved in microglial and astrocytic activation as well as in regulation of neuronal neuropeptides expression. There is evidence that IL-6 contributes to the development of neuropathic pain behavior following a peripheral nerve injury (Zhang & An, 2007). For example, sciatic cryoneurolysis, a sympathetically-independent model of neuropathic pain involving repeatedly freezing and thawing a section of the sciatic nerve, results in increased IL-6 immunoreactivity in the spinal cord (Zhang & An, 2007). In addition, intrathecal infusion of IL-6 induces tactile allodynia and thermal hyperalgesia in intact and nerve-injured rats, respectively (Zhang & An, 2007)

TNF-α, also known as cachectin, is another inflammatory cytokine that plays a wellestablished, key role in some pain models (Zhang & An, 2007). TNF acts on several different signaling pathways through two cell surface receptors, TNFR1 and TNFR2 to regulate apoptotic pathways, NFkB activation of inflammation, and activate stress-activated protein kinases (SAPKs). TNF-α receptors are present in both neurons and glia (Zhang & An, 2007). TNF-α has been shown to play important roles in both inflammatory and neuropathic hyperalgesia. Intraplantar injection of complete Freund's adjuvant in adult rats resulted in significant elevation in the levels of TNF-α, IL-1β, and nerve growth factor (NGF) in the inflamed paw (Zhang & An, 2007). A single injection of anti-TNF-α antiserum before the CFA significantly delayed the onset of the resultant inflammatory hyperalgesia and reduced IL-1β but not NGF levels. Intraplantar injection of TNF-α also produces mechanical and thermal hyperalgesia (Zhang & An, 2007). It has been found that TNF-α injected into nerves induces Wallerian degeneration and generates the transient display of behaviors and endoneurial pathologies found in experimentally painful nerve injury. TNF binding protein (TNF-BP), an inhibitor of TNF, is a soluble form of a transmembrane TNF-receptor (Zhang & An, 2007). When TNF-BP is administered systemically, the hyperalgesia normally observed after lipopolysaccharide (LPS) administration is completely eliminated. Intrathecal administration of a combination of TNF-BP and IL-1 antagonist attenuated mechanical allodynia in rats with L5 spinal nerve transaction (Zhang & An, 2007).

**QUESTION 2: When is wound healing referred to as ‘impaired’? And why?**

Chronic wounds are, by definition, wounds that have failed to progress through the normal stages of healing and therefore enter a state of pathologic inflammation (Menke *et al*., 2007). As a result, the healing process is delayed, incomplete, and does not proceed in a coordinated manner, subsequently resulting in poor anatomical and functional outcome (Menke *et al*., 2007). These wounds cause a major disability and are characterized by chronicity and frequent relapse. The differential diagnosis of the underlying etiology of a nonhealing wound is large, but most (~70%) ulcers are caused by ischemia, secondary to diabetes mellitus, venous stasis, and pressure (Menke *et al*., 2007).

There are no large-scale, population-based studies that examine the prevalence and economic cost of chronic wounds in the United States (Menke *et al*., 2007). The prevalence of the 3 major types of nonhealing wounds is estimated to be between 3 and 6 million in the United States, with patients 65 years and older accounting for 85%. Nonhealing wounds result in enormous health care expenditures with the total cost being estimated at more than $3 billion per year (Menke *et al*., 2007). None of the financial estimates take into account the amount of lost work time, decreased productivity, disability payments, nor the cost of rehabilitation (Menke *et al*., 2007).

In addition, the resultant psychosocial damage incurred by patients and their significant others, friends, and families is incalculable. Unfortunately, nonhealing wounds are prone to complications that not only effect the time to healing completion but also have a negative impact on the patients themselves (Menke *et al*., 2007). The complications of chronic wounds include functional limitations, infections, and malignant transformation. Functional limitations include gait changes and difficulty ambulating. Many patients have chronic pain that decreases their quality of life. Another large category of complications is related to infections (Menke *et al*., 2007). Cellulitis, abscess formation, osteomyelitis, gangrene, and even sepsis all may occur as a result of an infected wound. Furthermore, chronic wounds have the potential for malignant transformation (i.e., Marjolin’s ulcer). Lastly, foot ulcers are one of the most common causes of nontraumatic amputation (Menke *et al*., 2007).

**QUESTION 3: The role of oxidative stress in the development and progression of impaired wound healing.**

As discussed above, a delicate balance between the positive role of ROS and their deleterious effects is important for proper wound healing (Sanchez *et al*., 2018). Whereas production of ROS is essential to initiate wound repair, excessive amount of ROS generation is deleterious in wound healing. Ongoing oxidative stress, associated with lipid peroxidation, protein modification and DNA damage has been shown to impair wound healing processes via increased cell apoptosis and senescence (Sanchez *et al*., 2018). In physiological conditions, low levels of ROS production by NOX activation in neutrophils and macrophages are responsible for respiratory bursts during phagocytosis of the inflammatory phase (Sanchez *et al*., 2018). In contrast, as chronic inflammation develops in pathological conditions, NOX activation is exacerbated, which may lead to excessive production of ROS production, further accelerating inflammation and oxidative stress cellular damage (Sanchez *et al*., 2018). Clinical studies suggest that non-healing wounds are maintained in highly oxidizing environment, which lead to impaired wound repair (Sanchez *et al*., 2018). Clinical conditions such as tissue hypoxia and hyperglycemia are typically associated with highly oxidizing environments (Sanchez *et al*., 2018).

Hypoxic Wound

Whereas generation of ROS during the normal wound healing is related to NOX activation, the presence of hypoxia stimulates oxidant production by the electron transport chain (ETC) of the mitochondria mainly via complexes I and III (Sanchez *et al*., 2018). This observation is paradoxical, in the sense that superoxide is a product of the one-electron reduction of O2, which is reduced in hypoxia. ETC-derived ROS are transferred across the inter-membrane space to reach the cytosol where they act as second messengers (Sanchez *et al*., 2018). During hypoxia, mitochondria augment the release of ROS in the cytosol, which appears counter intuitive as O2 tension is reduced in the mitochondrial compartment (Sanchez *et al*., 2018). Hypoxia-induced mitochondrial ROS release has been shown to activate cell protection signaling through transcriptional and post-translational mechanisms (Sanchez *et al*., 2018).

In line, low oxygen levels leading to mitochondrial ROS production activate prolyl-4-hydroxylases. Prolyl-4-hydroxyases can induce hypoxia-inducible factor 1 (HIF-1) activation, which is involved in regeneration of lost or damaged tissue in mammals (Sanchez *et al*., 2018). In the microenvironment of early wounds, ischemia due to vascular disruption and high O2 consumption by immune competent cells can favor O2 depletion and hypoxia. Moreover, pathological conditions, such as diabetes, impair microvascular blood flow, thus aggravating tissue oxygenation, whereas temporary hypoxia after injury can be beneficial for wound healing, prolonged or chronic hypoxia delays wound healing (Sanchez *et al*., 2018). Impaired wound repair in hypoxic tissue has been related to the combination of mechanisms that increase ROS production and reduce antioxidant defenses (Sanchez *et al*., 2018).

Diabetic Chronic Wound

ROS production by several ROS-generating enzymes is elevated in diabetic wounds. Expression and activity of NOX, the major source of ROS in many cell types, are increased in response to hyperglycemia through activation of the receptor for advanced glycation end products (RAGE) (Sanchez *et al*., 2018). NOX activity is also increased downstream of hyperglycemia-induced protein kinase C (PKC) activation in smooth muscle and endothelial cells. Similarly, hyperglycemia-induced angiotensin II type 1 receptor AT1 activation increases expression of p47phox and enhances ROS production by NADPH oxidase (Sanchez *et al*., 2018). AT1 is expressed by several cell types in the wound, including myofibroblasts and keratinocytes. Expression and activity of H2O2-producing enzyme xanthine oxidase (XO) is also increased in diabetic mouse wounds and in response to high glucose levels (Sanchez *et al*., 2018).

One of the most important sources of ROS in diabetes is the mitochondrial electron transport chain. In line, hyperglycemia increases superoxide production by increasing the amount of pyruvate oxidation in the TCA cycle and consequently the availability of electron donors NADH and FADH2 (Sanchez *et al*., 2018). Increased electron flux then increases the proton gradient across the inner mitochondrial membrane, which at a critical threshold disrupts electron transport through complex III. Electron transport is then largely mediated by coenzyme Q, which transfers only one electron to oxygen, producing excess superoxide. Excessive mitochondrial superoxide production further impacts ROS levels by altering the flux through several intracellular pathways (Sanchez *et al*., 2018). For example, ROS leads to GAPDH inhibition by poly (ADP-ribose) modification, which increases levels of glycolysis intermediates upstream of GAPDH. This provides increased substrate levels for the polyol, protein kinase C, and hexosamine pathways. Activation and interaction of these pathways ultimately alters gene expression, depletes antioxidant resources, and favors the production of further ROS and advanced glycation end products (Sanchez *et al*., 2018). In addition, multiple lines of evidence have emerged showing that intracellular sites of ROS production are functionally connected. So-called ROS-induced ROS release cross talk represents a common mechanism for ROS amplification and regional ROS generation (Sanchez *et al*., 2018). A large number of mitochondrial pores (mPTP, inner membrane anion channel (IMAC), voltage dependent anion channels VDAC) has been identified as facilitating superoxide escape to the cytosol (Sanchez *et al*., 2018).

Hyperglycemia, mitochondrial ROS generation, and oxidative stress are involved in the pathogenesis of several diabetic complications. Deleterious effects of ROS on cellular homeostasis are also related to the reduction in antioxidant defenses, which intensifies the redox imbalance (Sanchez *et al*., 2018). Analysis of blood collected from diabetes patients showed reduced SOD, CAT, and glutathione peroxidase activity, and an overall decrease in antioxidant status. Of note, signaling through the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of antioxidant gene expression, is impaired in diabetes (Sanchez *et al*., 2018). Expression and nuclear translocation of Nrf2 are decreased in diabetic dermal fibroblasts. In response to oxidative stress, Nrf2 activity decrease was associated with reductions in expression of CAT, NADPH dehydrogenase quinone 1 (NOQ1), glutathione reductase, and glutathione S-transferase. In fibroblasts exposed to high glucose concentrations, Nrf2 is retained in the cytoplasm by its regulator Keap1, and transcription of MnSOD and NOQ1 is reduced (Sanchez *et al*., 2018). Activation of ATF-3 and NF-\_B is involved in antioxidant enzyme regulation is also altered in response to foot ulceration in diabetic patients (Sanchez *et al*., 2018).

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