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| ANA 404: INTRODUCTION TO HISTOPATHOLOGY |
| WOUND HEALING |
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QUESTION 1

Cytokines represent essential mediators of cell–cell communication with particularly important roles within the immune system. These secreted factors are produced in response to developmental and/or environmental cues and act via cognate cytokine receptors on target cells, stimulating speciﬁc intracellular signaling pathways to facilitate appropriate cellular responses. This review describes the evolution of cytokine receptor signaling, focusing on the class I and class II receptor families and the downstream JAK–STAT pathway along with its key negative regulators. Individual components generated over a long evolutionary time frame coalesced to form an archetypal signaling pathway in bilateria that was expanded extensively during early vertebrate evolution to establish a substantial “core” signaling network, which has subsequently undergone limited diversiﬁcation within discrete lineages. The evolution of cytokine receptor signaling parallels that of the immune system, particularly the emergence of adaptive immunity, which has likely been a major evolutionary driver.

Emergence of the pathway

Each of the core components of the CytoR signaling paradigm consists of modular protein domains, many of which have a long evolutionary history. The initial steps in the evolution of CytoR signaling involved the generation of the individual components of the pathway and their subsequent consolidation into a functional signaling system. The components arose largely by accretion of pre-existing domains across a broad evolutionary time frame. However, the generation of all components of the canonical CytoR–JAK–STAT module and their coalescence into a functional pathway only occurred in bilateria (Liongue *et al*., 2013).

Generating the components.

Individual components of the cytokine signaling pathway were assembled largely from preexisting domains that were modiﬁed for purpose. Cytokines, The ligands that activate class I and class II receptors are small polypeptides ∼5–25 kDa, which derive from a four helix–bundle structure that has been used in diverse proteins across evolution, from cytochromes to ferritin (Finn *et al*., 2016). The ﬁrst deﬁnitive representatives are present in extant bilateria, characterized by the fruit-ﬂy Unpaired proteins, which possess a so called “long” chain conformation most similar to vertebrate leptin and IL-6 (Harrison *et al*., 1998, Oldefest *et al*., 2013). CytoRs. Class I and class II receptors are cell surface protein complexes that consist of one to four receptor chains, at least one of which can transmit an intracellular signal to mediate the effects of the cytokine. These receptor chains possess an extracellular CytoR homology domain (CHD) consisting of two ﬁbronectin (FBN) type III folds with a connecting sequence associated with cytokine binding. They are divided into two families based on structural differences within the CHD: class I receptor chains possess two pairs of disulﬁde-linked cysteines within the ﬁrst FBN fold and a highly conserved WSXWS motif toward the C terminus of the second FBN fold (Bazan, 1990; Bagley, 1997; Lockyer, 2001), whereas class II receptor chains have one cysteine pair located in each FBN fold of their CHD (Bazan *et al*., 1990). The CytoR chains can also possess additional domains, including extracellular Ig-like and FBN-like regions, as well as transmembrane and intracellular sequences essential for signal transduction (Bazan, 1990; Kishimoto, 1994).

Diversiﬁcation of the pathway

Having established a core group of CytoR–JAK–STAT pathways, additional diversiﬁcation occurred during subsequent vertebrate evolution. Prior to the divergence of ray-ﬁnned ﬁsh (including teleost ﬁsh, such as zebraﬁsh) and lobe-ﬁnned ﬁsh (including tetrapods, such as humans), limited additional components evolved, with available data suggesting just the ligand-speciﬁc receptor chain for OSMR and the negative regulator SHP3, as well as a potential partial precursor of TSLPR, probably by local duplication. Along the tetrapod lineage, local duplication subsequently generated the distinct ligand-speciﬁc chains for IL-2R and IL-15R, as well as IL-3R and GM-CSFR, along with a bona ﬁde ligand-speciﬁc TSLPR chain (Boulay *et al*., 2003). There has been a concomitant increase in the cognate cytokines for these receptors (Boulay, 2003; Huising, 2006), whereas the diversity of type I and type III IFNs has also increased (Lutfalla, 2003; Krause, 2005). However, downstream components remain largely unaltered, with the exception of the duplication of a single STAT (STAT5) in mammals and loss of SHP3, which remains as a pseudogene (Liongue *et al*., 2012).

Emergence: The generation of individual CytoR–JAK–STAT pathway components largely by accretion of pre-existing domains and their subsequent coalescence into a complete signaling module. This archetypal pathway had diverse and pleiotropic functions, including a role in innate immunity. Expansion: The rapid increase in components during early vertebrate evolution principally driven by two rounds of WGD that ultimately produced core representatives for each of the major CytoR groups, along with their cognate ligands, and the majority of JAK, STAT, and key negative-regulatory components.

Cytokines in wound healing

The response to injury is a phylogenetically primitive, yet essential innate host immune response for restoration of tissue integrity. Tissue disruption in higher vertebrates, unlike lower vertebrates, results not in tissue regeneration, but in a rapid repair process leading to a fibrotic scar. Wound healing, whether initiated by trauma, microbes or foreign materials, proceeds via an overlapping pattern of events including coagulation, inflammation, epithelialization, formation of granulation tissue, matrix and tissue remodeling. The process of repair is mediated in large part by interacting molecular signals, primarily cytokines, that motivate and orchestrate the manifold cellular activities which underscore inflammation and healing.

Response to injury is frequently modeled in the skin(Singer *et al*., 1999), but parallel coordinated and temporally regulated patterns of mediators and cellular events occur in most tissues subsequent to injury. The initial injury triggers coagulation and an acute local inflammatory response followed by mesenchymal cell recruitment, proliferation and matrix synthesis. Failure to resolve the inflammation can lead to chronic nonhealing wounds, whereas uncontrolled matrix accumulation, often involving aberrant cytokine pathways, leads to excess scarring and fibrotic sequelae. Continuing progress in deciphering the essential and complex role of cytokines in wound healing provides opportunities to explore pathways to inhibit/enhance appropriate cytokines to control or modulate pathologic healing.

Most types of injury damage blood vessels, and coagulation is a rapid-fire response to initiate hemostasis and protect the host from excessive blood loss. With the adhesion, aggregation and degranulation of circulating platelets within the forming fibrin clot, a plethora of mediators and cytokines are released, including transforming growth factor beta (TGF-beta), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), that influence tissue edema and initiate inflammation. VEGF, a vascular permeability factor, influences the extravasation of plasma proteins to create a temporary support structure upon which not only activated endothelial cells, but also leukocytes and epithelial cells subsequently migrate (Liekens *et al*., 2001). Angiopoietin-1 (Ang-1), the ligand for Tie-2 receptors, is a natural antagonist for VEGF’s effects on permeability, a key regulatory checkpoint to avoid excessive plasma leakage.

Latent TGF-beta1, released in large quantities by degranulating platelets, is activated from its latent complex by proteolytic and non-proteolytic mechanisms (Khalil *et al*., 1999) to influence wound healing from the initial insult and clot formation to the final phase of matrix deposition and remodelling (Wahl *et al*.,1999). Active TGF-beta1 elicits the rapid chemotaxis of neutrophils and monocytes to the wound site (Wahl *et al*., 1987) in a dose-dependent manner through cell surface TGF-beta serine/threonine type I and II receptors and engagement of a Smad3-dependent signal (Ashcroft *et al*., 1999). Autocrine expression of TGF- beta 1 by leukocytes and fibroblasts, in turn, induces these cells to generate additional cytokines including tumor necrosis factor alpha (TNF-a), interleukin 1 beta (IL-1 beta) and PDGF, as well as chemokines, as components of a cytokine cascade (McCartney-Francais *et al*., 2001). Such factors act to perpetuate the inflammatory cell response, mediating recruitment and activation of neutrophils and monocytes. In response to TGF- beta and other cytokines, which engage their respective cell surface receptors, intracellular signaling pathways are mobilized to drive phenotypic and functional responses in target cell populations(Heldin *et al*., 2001). Among the upstream signaling cascades engaged in acute tissue injury are NF-?B, Egr-1, Smads, and MAP kinases, which result in activation of many cognate target genes, including adhesion molecules, coagulation factors, cytokines and growth factors (Heldin, 2001; Braddock, 2001).

Growth factors, cytokines and chemokines are crucial for coordinating multiple cell types during the healing process, making cutaneous wound healing possible. Proper wound healing is guided by stringent regulation of these agents as well as a wound environment that favors their activity. In the acute wound, the healing process is controlled by spatio-temporal action of these growth factors, cytokines and chemokines leading through progression of healing and resulting in the reestablishment of the skin’s barrier function. This is contrasted by the chronic wound, which is arrested in a state of chronic inﬂammation. As a consequence, the generation of a proteolytic environment by inﬂammatory cells inﬁltrating the wound site as well as prolonged up-regulation of pro-inﬂammatory cytokines and chemokines inhibits normal progression of wound healing. This environment subjects various growth factors and cytokines to degradation and sequestration in the wound site inhibiting their function. Topical delivery of growth factors to chronic wounds must be resistant to rapid degradation form the wounds proteolytic environment and have sustained release. This is readily being accomplished using gene therapy. Currently, multiple novel delivery systems, including adenovirus and slow-releasing polymers are being investigated as growth factor delivery systems. The most promising growth factors that require clinical testing are VEGF, bFGF, and GM-CSF. PDGF-BB has already been approved by the FDA and is currently used in the treatment of chronic ulcers. Living cell therapy, which is also FDA approved, may be considered as sustained, simultaneous multiple growth factor therapy. Both healthy keratinocytes and ﬁbroblasts produce at least 17 different growth factors (Brem *et al*., 2003) and secrete these factors stimulating patients’ cells to participate in healing process(Philips, 2002; Falanga, 2002). Despite these novel approaches, wound debridement should remain an integral component in treating chronic wounds. Debridement facilitates growth factor delivery by restoring the expression of growth factor receptors that are not properly expressed at the nonhealing edge of chronic ulcers, making cells unresponsive to exogenous growth factor therapy (Brem, 2007 & 2003).

QUESTION 2

In wounds where oxygenation is not restored, healing is impaired.

Oxygen is important for cell metabolism, especially energy production by means of ATP, and is critical for nearly all woundhealing processes. It prevents wounds from infection, induces angiogenesis, increases keratinocyte differentiation, migration, and re-epithelialization, enhances fibroblast proliferation and collagen synthesis, and promotes wound contraction (Bishop, 2008; Rodriguez *et al*., 2008). In addition, the level of superoxide production (a key factor for oxidative killing pathogens) by polymorphonuclear leukocytes is critically dependent on oxygen levels.

Due to vascular disruption and high oxygen consumption by metabolically active cells, the microenvironment of the early wound is depleted of oxygen and is quite hypoxic. Several systemic conditions, including advancing age and diabetes, can create impaired vascular flow, thus setting the stage for poor tissue oxygenation. In the context of healing, this overlay of poor perfusion creates a hypoxic wound. Chronic wounds are notably hypoxic; tissue oxygen tensions have been measured transcutaneously in chronic wounds from 5 to 20 mm Hg, in contrast to control tissue values of 30 to 50 mm Hg (Tandara and Mustoe, 2004).

In wounds where oxygenation is not restored, healing is impaired. Temporary hypoxia after injury triggers wound healing, but prolonged or chronic hypoxia delays wound healing (Bishop, 2008; Rodriguez et al., 2008). In acute wounds, hypoxia serves as a signal that stimulates many aspects of the wound-healing process. Hypoxia can induce cytokine and growth factor production from macrophages, keratinocytes, and fibroblasts. Cytokines that are produced in response to hypoxia include PDGF, TGF-β, VEGF, tumor necrosis factor-α (TNF-α), and endothelin-1, and are crucial promoters of cell proliferation, migration and chemotaxis, and angiogenesis in wound healing (Rodriguez *et al*., 2008).

In normally healing wounds, ROS such as hydrogen peroxide (H2O2) and superoxide (O2) are thought to act as cellular messengers to stimulate key processes associated with wound healing, including cell motility, cytokine action (including PDGF signal transduction), and angiogenesis. Both hypoxia and hyperoxia increase ROS production, but an increased level of ROS transcends the beneficial effect and causes additional tissue damage (Rodriguez et al., 2008).

Other factors that impair wound healing include:

Infections

Once skin is injured, micro-organisms that are normally sequestered at the skin surface obtain access to the underlying tissues. The state of infection and replication status of the micro- organisms determine whether the wound is classified as having contamination, colonization, local infection/critical colonization, and/or spreading invasive infection. Contamination is the presence of non-replicating organisms on a wound, while colonization is defined as the presence of replicating micro- organisms on the wound without tissue damage. Local infection/ critical colonization is an intermediate stage, with micro- organism replication and the beginning of local tissue responses. Invasive infection is defined as the presence of replicating organisms within a wound with subsequent host injury (Edwards and Harding, 2004).

Age

The elderly population (people over 60 years of age) is growing faster than any other age group (World Health Organization [WHO, www.who.int/topics/ageing]), and increased age is a major risk factor for impaired wound healing. Many clinical and animal studies at the cellular and molecular level have examined age-related changes and delays in wound healing. It is commonly recognized that, in healthy older adults, the effect of aging causes a temporal delay in wound healing, but not an actual impairment in terms of the quality of healing (Gosain and DiPietro, 2004; Keylock *et al*., 2008). Delayed wound healing in the aged is associated with an altered inflammatory response, such as delayed T-cell infiltration into the wound area with alterations in chemokine production and reduced macrophage phagocytic capacity (Swift *et al*., 2001). Delayed re-epithelialization, collagen synthesis, and angiogenesis have also been observed in aged mice as compared with young mice (Swift *et al*., 1999). Overall, there are global differences in wound healing between young and aged individuals. A review of the age-related changes in healing capacity demonstrates that every phase of healing undergoes characteristic age-related changes, including enhanced platelet aggregation, increased secretion of inflammatory mediators, delayed infiltration of macrophages and lymphocytes, impaired macrophage function, decreased secretion of growth factors, delayed re-epithelialization, delayed angiogenesis and collagen deposition, reduced collagen turnover and remodeling, and decreased wound strength (Gosain and DiPietro, 2004).

Stress

Stress has a great impact on human health and social behavior. Many diseases—such as cardiovascular disease, cancer, compromised wound healing, and diabetes—are associated with stress. Numerous studies have confirmed that stress-induced disruption of neuroendocrine immune equilibrium is consequential to health (Glaser and Kiecolt-Glaser, 2005; Vileikyte, 2007). The pathophysiology of stress results in the deregulation of the immune system, mediated primarily through the hypothalamic-pituitaryadrenal (HPA) and sympathetic-adrenal medullary axes or sympathetic nervous system (SNS; Godbout and Glaser, 2006; Boyapati and Wang, 2007).

Sex Hormones in Aged Individuals

Sex hormones play a role in age-related wound-healing deficits. Compared with aged females, aged males have been shown to have delayed healing of acute wounds. A partial explanation for this is that the female estrogens (estrone and 17β-estradiol), male androgens (testosterone and 5α-dihydrotestosterone, DHT), and their steroid precursor dehydroepiandrosterone (DHEA) appear to have significant effects on the wound-healing process (Gilliver *et al*., 2007). It was recently found that the differences in gene expression between elderly male and young human wounds are almost exclusively estrogen-regulated

(Hardman and Ashcroft, 2008). Estrogen affects wound healing by regulating a variety of genes associated with regeneration, matrix production, protease inhibition, epidermal function, and the genes primarily associated with inflammation (Hardman and Ashcroft, 2008). Studies indicate that estrogen can improve the age-related impairment in healing in both men and women, while androgens regulate cutaneous wound healing negatively (Gilliver *et al*., 2007).

Some more factors include:

Diabetes, medications (Glucocorticoid steroids, non-steroidal anti-inflammatory drugs, chemotherapeutic drugs), obesity, smoking, alcohol consumption

QUESTION 3

Wound healing is a well-tuned biological process, which is achieved via consecutive and overlapping phases including hemostasis, inﬂammatory-related events, cell proliferation and tissue remodeling. Several factors can impair wound healing such as oxygenation defects, aging, and stress as well as deleterious health conditions such as infection, diabetes, alcohol overuse, smoking and impaired nutritional status. Growing evidence suggests that reactive oxygen species (ROS) are crucial regulators of several phases of healing processes. ROS are centrally involved in all wound healing processes as low concentrations of ROS generation are required for the ﬁght against invading microorganisms and cell survival signaling. Excessive production of ROS or impaired ROS detoxiﬁcation causes oxidative damage, which is the main cause of non-healing chronic wounds. In this context, experimental and clinical studies have revealed that antioxidant and anti-inﬂammatory strategies have proven beneﬁcial in the non-healing state. Among available antioxidant strategies, treatments using mitochondrial-targeted antioxidants are of particular interest. Speciﬁcally, mitochondrial-targeted peptides such as elamipretide have the potential to mitigate mitochondrial dysfunction and aberrant inﬂammatory response through activation of nucleotide-binding oligomerization domain (NOD)-like family receptors, such as the pyrin domain containing 3 (NLRP3) inﬂammasome, nuclear factor-kappa B (NF-κB) signaling pathway inhibition, and nuclear factor (erythroid-derived 2)-like 2 (Nrf2).

A delicate balance between the positive role of ROS and their deleterious effects is important for proper wound healing. Whereas production of ROS is essential to initiate wound repair, excessive amount of ROS generation is deleterious in wound healing. On going 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 (Sen, 2008; Schafer, 2008; Dunnill, 2017; Sen, 2009; Bryan, 2012 ). 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 (Hoffman, 2018; Jiang, 2011; Levigne, 2016). 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. Clinical studies suggest that non-healing wounds are maintained in highly oxidizing environment, which lead to impaired wound repair. Clinical conditions such as tissue hypoxia and hyperglycemia are typically associated with highly oxidizing environments.

Hypoxic Wound WhereasgenerationofROSduringthenormalwoundhealingisrelatedtoNOXactivation (Hoffman, 2018; Jiang, 2011; Levigne, 2016), the presence of hypoxia stimulates oxidant production by the electron transport chain (ETC) of the mitochondria mainly via complexes I and III (Waypa, 2016). 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. During hypoxia, mitochondria augment the release of ROS in the cytosol, which appears counterintuitiveasO2 tension is reduced in the mitochondrial compartment (Waypa, 2016; Fuhrmann, 2017). Hypoxia-induced mitochondrial ROS release has been shown to activate cell protection signalling through transcriptional and post-translational mechanisms (Waypa, 2016; Fuhrmann, 2017). Inline, low oxygen levels leading to mitochondrial ROS production activate prolyl-4-hydroxylases. Prolyl-4-hydroxyases can induce hypoxia-inducible factor1 (HIF-1) activation, which is involved in regeneration of lost or damaged tissue in mammals (Fuhrmann, 2017; Wautier, 2017). In the microenvironment of early wounds, ischemia due to vascular disruption and high O2 consumption by immune competent cells can favour O2 depletion and hypoxia (Guo, 2010; Sen, 2009). Moreover, pathological conditions, such as diabetes, impair microvascular blood ﬂow, thus aggravating tissue oxygenation (Guo *et al*., 2010), whereas temporary hypoxia after injury can be beneﬁcial for wound healing, prolonged or chronic hypoxia delays wound healing. Impaired wound repair in hypoxic tissue has been related to the combination of mechanisms that increase ROS production and reduce antioxidant defenses (Sen *et al*., 2009)

Diabetic Chronic Wound ROS production by several ROS-generating enzymes is elevated in diabetic wounds (Golebiewska *et al*., 2015). 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 (Shah *et al*., 2016). NOX activity is also increased downstream of hyperglycemia-induced protein kinase C (PKC) activation in smooth muscle and endothelial cells (Schramm *et al*., 2012) . Similarly, hyperglycemia-induced angiotensin II type 1 receptor AT1 activation increases expression of p47phox and enhances ROS production by NADPH oxidase (Kurosaka *et al*., 2009). AT1 is expressed by several cell types in the wound, including myoﬁbroblasts and keratinocytes (Fernandez *et al*., 2018). Expression and activity of H2O2-producing enzyme xanthine oxidase (XO) is also increased in diabetic mouse wounds and in response to high glucose levels (Forrester *et al*., 2018).

One of the most important sources of ROS in diabetes is the mitochondrial electron transport chain (Schramm *et al*., 2012). 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 (Schramm, 2012; Zinkevich, 2011). Increased electron ﬂux then increases the proton gradient across the inner mitochondrial membrane, which at a critical threshold disrupts electron transport through complex III (Zinkevich *et al*., 2011). Electron transport is then largely mediated by coenzyme Q, which transfers only one electron to oxygen, producing excess superoxide (Zinkevich *et al*., 2011). Excessive mitochondrial superoxide production further impacts ROS levels by altering the ﬂux through several intracellular pathways. For example, ROS leads to GAPDH inhibition by poly(ADP-ribose)modiﬁcation, which increases levels of glycolysis intermediates upstream of GAPDH (Schramm, 2012; Zinkevich, 2011). This provides increased substrate levels for the polyol, protein kinase C, and hexosamine pathways (Schramm, 2012; Zinkevich, 2011). 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. 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 (Zorov *et al*., 2014). 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 (Zorov, 2014; Banerjee, 2014). 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. Analysis of blood collected from diabetes patients showed reduced SOD, CAT, and glutathione peroxidase activity, and an overall decrease in antioxidant status (David *et al*., 2017). 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 (Bitar *et al*., 2011).

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 (Soares *et al*., 2016). 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 (Ambrozova *et al*., 2017). Activation of ATF-3 and NF-κB is involved in antioxidant enzyme regulation is also altered in response to foot ulceration in diabetic patients (Wang *et al*., 2017).

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