Delayed Wound Healing: A Focus on Bacterial Intracellular Proteases in Diabetic Wound Ulcers

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ABSTRACT

A diabetic skin ulcer (DSU) is a breach on the skin characterized by progressive destruction of the surface epithelium of the skin and a disintegrating base resulting from wounds obtained by a diabetic patient. This study was aimed at determining DSU wound colonization by bacteria and to quantitatively analyze some intracellular enzymes produced by these bacteria that could affect the wound healing process. Wound swab samples were collected from 150 subjects comprising of diabetic patients with skin ulcers as well as non-diabetics with wounds (control). The wounds were found to be colonized by an array of bacteria which included Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter sp, Enterococcus faecalis, Proteus vulgaris, Proteus mirabilis, Klebsiella pneumoniae, Escherichia coli, Coagulase negative Staphylococcus aureus, Klebsiella oxytoca, Alcaligenes faecalis and Citrobacter freudii. These bacterial isolates produced the following proteolytic enzymes: collagenase, caseinase, hyaluronidase, alkaline protease and gelatinase on the DSU in higher amounts than the non-diabetic wounds (control). Intracellularly, S.aureus (0.199 ± 0.000) and E.coli (0.145 ± 0.006) secreted the maximum and minimum amounts of hyaluronidase respectively. For caseinase, S.aureus (0.048 ± 0.001) exhibited the highest activity and K.oxytoca (0.018 ± 0.000) showed the least activity. E.coli (18.173 ± 0.157) secreted the highest amount of gelatinase while P.aeruginosa (5.057 ± 0.496) had the least while maximum intracellular activity for alkaline protease was secreted by P.aeruginosa (0.016 ± 0.001). Bacterial wound flora was found capable of producing and secreting intracellular proteolytic enzymes which could be implicated in impairing proper wound healing.

Keywords: Diabetic Wounds, Intracellular Bacterial Proteases, Wound Healing

INTRODUCTION

Diabetic skin ulcers are the most dreadful and difficult to treat complications of diabetes mellitus. Diabetic skin ulcers evolve in about 15 percent of people with Diabetes mellitus and have become the principal proximate trigger for non-traumatic extremity amputations throughout the world. An estimated 85 percent (85%) of amputation cases are due to diabetic skin ulcers.[1] In Nigeria, prevalence of diabetic skin ulcer ranges from 0.8-11 % in urban and rural populations.[2]

A diabetic skin ulcer (DSU) is defined as a breach on the skin characterized by progressive destruction of the surface epithelium of the skin and a disintegrating base resulting from wounds obtained by a diabetic.[3] It involves various pathological complications including peripheral vascular disease, neuropathy, ulceration of wounds and infections (with or without osteomyelitis) which lead to gangrene development and may even necessitate amputation of the limb. DSUs can even result in considerable morbidity and mortality of the patient due to increased susceptibility to infection and impaired wound healing. A major hindrance in the controlling of DSU is the colonization of wounds by bacterial pathogens.[4]

Diabetes decelerates the normal functioning process of wound healing, leading to vascular and neuropathic disorders. The microbes are therefore able to overcome the host's cell barrier due to the degradation of cell epithelium resulting from the peripheral neuropathy associated with Diabetes.[5] Also, increased hyperglycemia activates advanced glycation of lipids and proteins which accumulate in the capillaries, thus resulting in decreased flow of blood to the infected wound site.[6] This impaired circulation in micro-vascular system in diabetics with wounds hinders the phagocytes' access thus enhancing

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the development of an infection.[5] Hence, increased susceptibility to infection is found in diabetic patients, rather than non-diabetic persons.

Bacteria produce proteolytic enzymes such as collagenase, gelatinase, caseinase, alkaline protease hyaluronidaseetc, which locally damage the cells of the host and aid the pathogenic bacteria in their spread.[5,7-9]These enzymes play a significant role in impairing wound healing because bacterial proteases can cause the destruction of collagen, cell membrane and muscle fibres and increase capillary permeability for the establishment of the infecting bacteria. They hydrolyze the proteins and peptides and therefore cause cell membrane degradation and disruption of various biological activities.[5] The recognition of the organisms and their biochemical parameters will help provide better treatment for the diabetic skin ulcer problem and other inadequately healing wounds.[10] Even though the diabetic skin ulcer is a multi-factorial condition, one of the most significant reasons for the non-healing nature of the wound is the destruction of tissues caused by the increased bacterial protease activity.[5]

Hence, the aim of this study was to determine the presence and effects of some intracellular proteolytic enzymes of bacterial origin on tissue damage and impaired wound healing in diabetic skin ulcers.

MATERIALS AND METHODS

A total of 150 subjects were recruited in this study. Wound ulcer swab samples were collected from diabetic skin ulcer patients as well as non-diabetics with wounds (control) in the Central Hospital Benin, St Philomena Hospital, Benin, Stella Obasanjo Hospital, Benin and the Federal Medical Centre, Asaba, Nigeria. Informed consent and ethical approval were obtained from the Institutes' Ethics Committee/ Hospitals' Management Board. The samples were cultured and bacterial isolates were obtained and were identified using standard bacteriological procedures. A quantitative study of the intracellular enzymes collagenase, caseinase, hyaluronidase, alkaline protease and gelatinase was carried out.

Isolated bacteria were separately inoculated into 10ml of nutrient broth and incubated for 24hr at 37°C. Each cultured isolate was centrifuged, and the supernatant and pellet were collected separately. The pellets were then suspended in 1ml phosphate buffer,sonicated and centrifuged and were used for the intracellular enzyme assay.

Collagenase and caseinase were assayed using the procedure.[11]A unit of the enzyme activity was described as the µM of L-leucine liberated in 5hr at culture conditions. Hyaluronidase assay was done using the methods of Tolksdorf and McCready, and Kass and Seastone.[12,13] The activity of alkaline protease was determined by the method of Meyers and Ahearn.[14] One unit of alkaline protease activity was expressed as the amount of the enzyme which released 1µmol tyrosine per ml per min.

Gelatinase activity was determined using the method described by Tran and Nagano and it was defined as the µM of leucine released per ml per min.[8]

RESULTS

The frequency of occurrence of bacterial isolates in diabetic skin ulcers and non-diabetic wounds is shown in table 1. Among the total bacterial isolates obtained, 34.78% of the isolates were Gram-positive, while 65.22% were Gram-negative. Among the Gram-positive bacteria, *Staphylococcus aureus* was the most predominant isolate (18.12%). Coagulase negative Staphylococcus (CONS) was the least isolated (2.17%). Among the Gram-negative bacteria, *Escherichia coli* was the most common isolate (17.39%) and *Acinetobacter* species was the least common bacterial isolate (2.17%). A higher percentage of the bacterial isolates also occurred in the diabetic skin ulcers than the non-diabetic wounds.

A profile of the different proteolytic enzymes produced by the bacterial isolates from diabetic skin ulcers is shown in table 2. All isolates secreted intracellular gelatinase which had 100% occurrence. Alkaline protease was the least secreted enzyme intracellularly. Intracellular enzyme

activities on the wounds were observed as follows: collagenase (81.88%), caseinase (86.23%), hyaluronidase (69.56%), alkaline protease (54.35%) and gelatinase (100%).

A comparative analysis of the intracellular proteases secreted by bacterial isolates in diabetic skin ulcers and non-diabetic wounds of studied subjects is shown on table 3. The values obtained revealed that higher quantities of the intracellular proteases were secreted by the bacterial isolates in the diabetic ulcers than those in the non-diabetic wounds.

Table 1: Frequency of occurrence of bacterial isolates in diabetic skin ulcers and non-diabetic wounds.

Bacteria	Diabetic Skin Ulcers n=138 (%)	Non-Diabetic Wounds n=18 (%)
Gram Positive		
Staphylococcus aureus	25(18.12)	4(22.22)
Enterococcus faecalis	20(14.49)	-
Coagulase negative staphylococcus (CONS)	3(2.17)	-
Gram Negative		
Escherichia coli	24(17.39)	6(33.33)
Klebsiellapneumoniae	13(9.42)	2(11.11)
Proteus mirabilis	12(8.70)	1(5.56)
Pseudomonas aeruginosa	10(7.25)	2(11.11)
Klebsiellaoxytoca	10(7.25)	-
Citrobacterfreundii	7(5.07)	-
Proteus vulgaris	7(5.07)	-
Alcaligenesfaecalis	4(2.90)	3(16.67)
*Acinetobacter*sp.	3(2.17)	-

Table 2**:** Profile of intracellular protease enzymes produced by different bacterial isolates on diabetic skin ulcers.

	Intracellular Enzymes (Mean ± SEM)				
Bacteria	**Collagenase (unit/ml/min)**	**Caseinase (unit/ml/min)**	**Hyaluronidase (mg HA digested)**	**Alkaline Protease (unit/ml/min)**	**Gelatinase (unit/ml/min)**
S.aureus	0.018±0.001	0.048±0.001	0.199±0.000	0.007±0.000	180.054±0.363
E.faecalis	0.025±0.003	0.045±0.001	0.164±0.012	-	160.279±0.016
CONS	0.016±0.006	0.026±0.012	0.183±0.005	0.006±0.001	60.096±0.065
E.coli	0.018±0.007	0.033±0.007	0.145±0.006	-	180.173±0.157
K.pneumoniae	0.017±0.001	0.039±0.008	-	0.004±0.002	135.032±0.132
P.mirabilis	-	-	-	-	143.228±0.069
P.aeruginosa	-	0.046±0.001	-	0.016±0.001	50.057±0.496
K.oxytoca	0.009±0.001	0.018±0.000	0.162±0.015	0.012±0.001	129.760±0.345
C.freundii	0.011±0.001	0.040±0.001	0.186±0.002	0.011±0.002	120.087±0.036
P.vulgaris	0.020±0.006	-	-	-	150.069±0.008
A.faecalis	0.009±0.001	0.043±0.001	0.174±0.012	0.009±0.001	122.005±0.081
***Acinetobacter*sp.**	-	0.045±0.001	0.168±0.008	0.005±0.000	130.274±0.017

Table 3: Comparative profile of intracellular enzymes secreted by bacterial isolates from diabetic skin ulcers and non-diabetic wounds.

Intracellular Enzymes	Diabetic Skin Ulcer	Non-Diabetic Wounds
COLLAGENASE (mg/ml/min)	0.009±0.001 - 0.025±0.003	0.002±0.000 - 0.009± 0.001
CASEINASE (mg/ml/min)	0.018±0.000 - 0.048±0.001	0.009±0.002 - 0.017±0.002
HYALURONIDASE (mgHA digested)	0.145±0.006 - 0.199±0.000	0.034±0.003 - 0.118±0.025
ALKALINE PROTEASE (mg/ml/min)	0.004±0.002 - 0.016±0.001	-
GELATINASE (mg/ml/min)	5.057±0.496 - 18.173±0.157	0.129±0.007 - 3.024±0.401

DISCUSSION

The array of bacteria isolated from the wounds included *Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobactersp, Enterococcus faecalis, Proteus vulgaris and Proteus mirabilis, Klebsiella pneumoniae, Escherichia. coli*, Coagulase negative *Staphylococcus aureus*, *Klebsiella oxytoca, Alcaligenes faecalis* and *Citrobacter freudii*. Bacterial colonization limits the healing process of the wound. Therefore, these bacterial pathogens cause disease by establishment mechanisms, invasins production and evading the defense mechanisms of the host.

This study revealed that these bacteria produced some intracellular proteolytic enzymes. These enzymes play very significant roles in making the wounds worse. Bacterial protease cause deterioration of the cell membranes and disrupt several biological functions by hydrolyzing the proteins and peptides of the cells.[5]From the results obtained, it was observed that the enzyme intracellular collagenase was secreted by all isolated bacteria except *Proteus mirabilis, Pseudomonas aeruginosa* and *Acinetobacter sp*. The high rate at which collagenase was secreted by the bacterial isolates depicts its ability in causing uncontrolled proteolytic destruction of tissues, thus acting as a pathogenic factor in the non-healing wound. Due to the abundance of collagen in the skin, bones and other connective tissues, bacterial collagenase which tend to break down the on-site collagen can bring about uncontrolled proteolytic destruction of tissues and pose as a virulence factor in the unhealing wounds, resulting in ulceration of the skin and osteomyelitis of the bones.

A few of the organisms under consideration gave intracellular activities for hyaluronidase and this enzyme functions as a virulence factor and is responsible for the breaking down of cell membrane polysaccharides and thus, increasing the permeability of the cell wall, which enhances bacterial spread around the wound surface.[15]

The presence of the enzyme caseinase has been detected in many hospital clinical isolates such as those obtained from respiratory tract secretions, in corneal ulceration during bacterial keratitis and also in some strains. The proteolytic activity of the enzyme has also been found to be related with the pathogenesis of the producing bacteria and the development of hospital-acquired infections and also essential for the activity of haemolysin.[5] Hence, the occurrence of caseinase-producing bacteria in diabetic skin ulcer has an influence in delayed diabetic skin ulcer management.

All bacterial isolates in this study were observed to secrete gelatinase intracellularly. Bacterial gelatinase breaks down gelatin present in connective tissues and aid in the continuous spread of the microorganisms into the tissues.[5]Thus, they might play a role making diabetic skin ulcers worse.

Alkaline proteases affect the wound healing process by causing an increase in the pH at the site of the wound. Normal wound healing process readily occurs in an acidic environment between pH 4-6. The enzyme acts by elevating the wound's pH, and therefore affects many functions like

angiogenesis, release of oxygen, bacterial toxicity, protease activity etc., thus causing the wound to stay unhealed.[9] Alkaline proteases cleave protein peptide bonds and are usually stable at a higher or alkaline pH. Alkaline proteases also exhibit proteolytic activity on proteins that are involved in the defense mechanism of the host, such as the activation of complement through the classical and the lectin pathways and also the penetration of the barriers of the body, causing damage to the cells of the host. They also help the organism to evade the host's immune system by protecting them. Bacterial alkaline protease can cause these effects even in very small amounts.[5] This supports the findings of the present study because organisms positive for alkaline protease were significantly less. Although the activity of the enzyme was less, it has severe consequences on already debilitating conditions of diabetic skin ulcers hence greatly impairing the wound healing process.[5]

In comparing the diabetic skin ulcers and the non-diabetic wounds, it was observed that much higher amounts of all the proteolytic enzymes secreted (collagenase, caseinase, hyaluronidase, alkaline protease and gelatinase) were observed in diabetic skin ulcers. This indicates that the presence of these proteases can be implicated in the slow healing process of diabetic skin ulcers compared to non-diabetic wounds.

CONCLUSION

This study suggests that bacterial enzymes play a major role in tissue damage and impaired wound healing associated with diabetic skin ulcers, thus providing information to help produce better treatment options for diabetic skin ulcers.

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