

Original Article

STUDY OF MORPHOLOGY OF PLATELETS IN NORMAL AND PREMALIGNANT LESIONS USING PHASE CONTRAST MICROSCOPY

Treville Pereira, Ashwini Pujar, Avinash Tamgadge

Dept of Oral Pathology and Microbiology, Dr D Y Patil Dental College & Hospital, Sector 7, Nerul, Navi Mumbai, Maharashtra, India, Pincode- 400706

ABSTRACT:

Background: Platelets are small irregularly shaped clear cell fragments, which are derived from the fragmentation of precursor of megakaryocytic cells. However the interaction between platelets and cancer cells are less appreciated. This is surprising considering the fact that blood vessels are the major anatomical pathways for cancer cell dissemination. Phase contrast microscopy is a method that enables us to see very transparent objects which are invisible by ordinary, light microscopy. The aim was to study the morphology of human blood platelets in normal and precancer patients and to correlate this with the degree of dysplasia. The platelets were suspended in hypotonic sodium chloride (NaCl) solution at various concentrations. Platelets underwent marked morphologic changes with decreasing concentration of salt solutions and these changes were observed by means of phase contrast microscopy. **Materials and methods:** The study group included 15 healthy patients and 15 patients with oral precancer aged between 19 to 62 years who had visited to the department of oral pathology during the period of July to October 2012. After histopathological confirmation blood was collected, centrifuged and the platelet suspension was extracted. One drop of platelet suspension in salt solution of varying concentration was placed between an ordinary slide and cover glass and immediately photographed. **Results:** Using the phase contrast microscopy normal human platelets have spider like appearance and also shows spicules on them at lower concentrations of NaCl solutions. In premalignant lesions platelets showed variation in the size of platelets, increase in number and no spider like process or spicules were observed. Few cases of premalignant lesions showed an increase of aggregation of platelets with increase in the degree of dysplasia. **Conclusion:** Platelet research in precancer patients is growing in investigation and could provide new pathophysiological insights.

Keywords: Premalignant, sodium chloride solution, phase contrast microscopy, platelets.

Corresponding Author: Dr. Treville Pereira, Dept of Oral Pathology and Microbiology, Dr D Y Patil Dental College & Hospital, Sector 7, Nerul, Navi Mumbai, Maharashtra, India, Pincode- 400706
Email: trevillepereira@gmail.com

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INTRODUCTION:

Platelets were first described by the remarkably early observations of Bizzozero in the late 1800s.^[1] Not only did he identify platelets as distinct corpuscles within human blood but he observed them forming thrombi within damaged areas of vessel wall using real time microscopy. Today, modern imaging methods are utilized to study in detail, the same real interactions of platelets with the vessel wall and dynamics of thrombus formation.^[2]

Platelets are cytoplasmic fragments of megakaryocytes (a type of white blood cell), which are formed in the marrow, and are round or oval in shape, approximately 2 μm in diameter.^[3] They have a trilaminar cell membrane with a glycoprotein receptor surface overlying and

partially interspersed with and penetrating a bilayer of phospholipids and cholesterol.^[4] Platelets lack nuclei but contain organelles and structures such as mitochondria, microtubules, and granules (α , δ and λ).^[4] There are approximately 50 to 80 α -granules per platelet, each bound by a unit membrane and formed during megakaryocyte maturation. The α granules are approximately 200 to 500 nm in diameter and contain over 30 bioactive proteins, many of which have a fundamental role in homeostasis and/or tissue healing.^[5] Platelets reside intravascularly and are concentrated in the spleen.^[3] The normal concentration of platelets in blood is approximately 140,000 to 400,000 platelets/mm.^[3] These remain in the circulation for an average of

approximately 10 days before removal by macrophages of the reticuloendothelial system.^[3,4]

Platelets are also surprisingly multifunctionally involved in many pathophysiological processes including homeostasis and thrombosis, clot retraction, vessel constriction and repair, inflammation including promotion of atherosclerosis, host defense and even tumor growth/metastasis.^[6]

Platelets may contribute to cancer progression by stabilizing tumor cell arrest in the vasculature, stimulating tumor cell proliferation, promoting tumor cells extravasation by potentiating tumor cell induced endothelial cell retraction, enhancing tumor cell interaction with the extracellular matrix.^[7]

In the present study, we have observed the morphology of human blood platelets in normal and precancer patients which were suspended in hypotonic Sodium chloride (NaCl) solutions at various concentrations. The NaCl solution helps in enlargement of the platelet cell which would help in appreciation of its morphological alteration. Literature search has shown that no study has not been carried out before on platelets in precancer patients. So, we decided to study the morphology of human blood platelets in precancer patients under phase contrast microscopy and to correlate this with the degree of dysplasia.

MATERIALS AND METHOD:

The study group comprised of 15 normal healthy patients and 15 patients with precancer lesions out of which 12 were oral submucous fibrosis (OSMF) and 3 were leukoplakic lesions. Approval for the study was obtained from the ethical review board of the institute and a written informed consent was signed by the patients. The study was carried out over a period of three months. Thorough screening and clinical examination for each patient was done. Healthy individuals without any oral lesions, habits or systemic diseases served as controls. The subjects with OSMF and leukoplakia had betel nut chewing habits over a period of 15 years. All the subjects were of a similar age group (ranging from 19 to 62 years). Provisional diagnosis of OSMF and leukoplakia was made on clinical examination, the confirmation of which was done by incisional biopsy and histopathological examination. The Haematoxylin and Eosin (H and E) stained sections were assessed by a single trained observer and graded according to the criteria used by Arne Burkhardt^[8] as mild, moderate and severe epithelial dysplasia for the cases of leukoplakia. The cases of OSMF were categorized depending on

the connective tissue changes into very early, early, moderately advanced and advanced stages according to Sirsat and Pindborg.^[9] After the histopathological confirmation 2ml of blood was taken using a sterile syringe from the antecubital fossa. The other materials used for the present study were Triton X100 solution, disodium sequesterene, sodium chloride solution along with a centrifugation machine, test tubes, glass slide, cover slip and phase contrast microscope.

Uses of solutions:

Triton and Disodium sequesterene solution is mainly used to prevent coagulation and irreversible clumping of platelets during centrifugation. NaCl helps in enlargement of cell which would help in appreciation of morphological alterations of platelets.^[10]

Glassware containing a solution composed of 1 % disodium sequesterene and 1% triton X 100 solution was prepared in 0.7 % sodium chloride. 1 ml of this solution was added to 2ml of blood to prevent both coagulation and irreversible clumping of platelets during centrifugation.

Preparation of platelet suspension:

Blood was withdrawn using a 2ml syringe and then 1 ml of the prepared solution containing the disodium sequesterene, triton X 100 and sodium chloride solution was added to it. The blood was centrifuged for 10 minutes. The supernatant plasma, containing a pure suspension of thrombocytes, was then centrifuged for 15 minutes to obtain sediment of blood platelets. To this platelet sediment prepared from 2 ml of blood, 1ml of normal saline was added and the tube, after standing for 30 minutes at 6°C, was gently shaken every 10 minutes to obtain a homogenous concentrate of platelets.

Preparation of Sodium chloride dilutions:

Serial dilution of saline from 0.60, 0.44, 0.34, 0.85 % were prepared in ten clean dry test tubes. 1ml of each solution was transferred to a test tube of the same size for the examination of each platelet sample.⁽⁷⁾

15 platelet samples obtained from normal healthy individuals and from precancer patients were examined for their osmotic fragility. To each test tube of saline, 0.05 ml of the platelet suspension was added with a pipette. The tubes were then gently shaken by hand to ensure homogenous distribution of platelets, and left at room temperature. Further, the test tubes were gentle

shaken every 30 minutes. The results were read after 2 hours. Before observing the findings, the tubes were again gently shaken and one drop was transferred with a pipette to a glass slide and covered with a cover slip. This preparation was examined immediately under oil immersion of a phase contrast microscope. The results were assessed by two trained observers.

RESULTS:

At a concentration of 0.85% NaCl, the platelets were fairly uniform and larger in size and the internal structure could be recognized (Figure1).

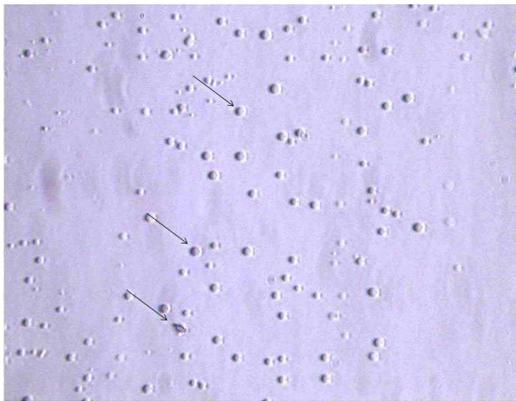


Figure 1: Normal platelets observed in phase contrast microscopy using 0.85 per cent sodium chloride solution. Platelets showed variation in size with no spicules.

At a concentration of 0.34%, about one third of the platelets was enlarged, rounded, and had a sharp process which was about five times longer than the whole platelet (Figure 2). Some platelets had two such processes which were somewhat smaller and thinner. These sword like processes were always clear and without any granulation. As the salt concentration decreased, the platelets tended to form aggregates of 5 to 10 cells. At a concentration of 0.34 % NaCl most of the cells had this appearance.



Figure 2: Normal platelets observed in phase contrast microscopy using 0.34 per cent of sodium chloride

solution. Platelets showed variation in size and spicules were seen on few of them.



Figure 3: Platelets observed in oral submucous fibrosis lesion and platelets showed variation in size, number and aggregates of 5 to 6 in number.



Figure 4: Platelets observed in leukoplakia and show variation in size, number and aggregates of 9 to 10 in number.

Platelets observed in OSMF and leukoplakia lesions , at a concentration of 0.34 and 0.85% platelets showed an increase or decrease in number, variation in size and platelets tended to form aggregates of 8 to 10 cells with no granulation, sword like process or any spicules (Figure 3,4).

DISCUSSION:

Platelets are small, specialized blood cells that are released as anuclear cytoplasmic bodies from megakaryocytes in the bone marrow. One litre of blood contains about 400 billion circulating platelets that are turned over every week.^[11] The platelet membrane consists of phospholipids and is covered with glycoproteins and integrins, which are essential for adhesion, aggregation and activation, the critical steps in platelet-mediated hemostasis. Platelet membrane receptors include Glycoprotein Ib-IX-V (GPIb-IX-V), Glycoprotein VI (GPVI) and Glycoprotein IIb-IIIa (GPIIb-IIIa, also as integrin α Ibb3), receptors that are essential for complete adhesion and aggregation.^[12] GPVI surface receptor, a member of the immunoglobulin

superfamily, which principally binds collagen, has become a subject of active investigation. A 50% reduction in experimental pulmonary metastasis in GPVI-deficient mice has been reported by Jain et al.^[13] Additional important receptors found on platelet membranes include the protease-activated receptors (PAR), PAR-1 and PAR-4, and the P2 receptors, P2Y1 and P2Y12, which principally mediate activation and aggregation. Platelets also contain three types of granules: (i) dense granules containing platelet agonists such as serotonin and ADP that serve to amplify platelet activation, (ii) a granules containing proteins that enhance the activation process and participate in coagulation; and (iii) lysosomal granules containing glycosidases and proteases.^[14] Many of the major structural components of platelets and platelet receptors that contribute to hemostasis have also been found to relate to malignancy progression. Platelets also store proteins within the alpha granule that can regulate angiogenesis and metastases. Further, platelet receptors such as GPIIb/IIIa can mediate platelet angiogenic protein release in addition to their more traditional role in fibrinogen binding. At least one study has found ultrastructural changes in platelets from patients with lung cancer, including an increase in the number of platelet alpha granules.^[15] In cellular models of both breast and ovarian cancer, invasiveness had increased following exposure to platelets.^[16] Platelet adhesion receptors play a critical role in tumor-platelet cross-talk and in the process of hematogenous metastasis.^[17] Functionally, platelets are complex cells capable of shape change, translational protein production, protein and metabolite release, cell-cell interactions and paracrine regulation. Most of these functions relate to the processes of platelet activation and aggregation that occur following exposure to an *in vivo* stimulus. In malignancy, tumor cells can activate platelets by direct contact, or via release of mediators such as ADP, thrombin, thromboxane A2 or tumor-associated proteinases. The relative importance of each platelet activator in malignancy is unknown and some data suggest the mechanism of platelet activation by tumor cells may be tumor cell specific and, in some cases, mutually exclusive. Platelets contribute to critical steps in cancer metastasis, including facilitating tumor cell migration, invasion and arrest within the vasculature. An important step in metastatic dissemination is the breakdown of vessel basement membrane. By releasing proteolytic enzymes such as gelatinase, heparanase and various matrix metalloproteinases (MMPs), activated platelets can directly degrade structural components, or

alternatively, support this process by activating other proteinases and/or enabling tumor cells and endothelial cells to do the same. Moreover, modulation of proteolytic activity is accomplished by growth factors released by platelets. Tumor cells have the ability to aggregate platelets, a finding first reported in 1968, and referred to as tumor cell-induced platelet aggregation (TCIPA).^[18] It is now recognized that this aggregation correlates with the metastatic potential of cancer cells in both *in vitro* and *in vivo* models of experimental metastasis. The mechanisms by which tumor cells induce platelet aggregation may differ by cancer type, but have in common the theme of conferring survival advantage. In turn, platelets can protect tumor cells in at least two ways: by coating them and thereby directly shielding them from physical stressors within the vasculature and by permitting evasion from the immune systems effector cells.^[18] In the present study aggregates of platelets were observed in the subjects who had precancer lesions.

Hematogenous dissemination is a hallmark of metastatic disease, and is the least controllable aspect of cancer leading to a fatal outcome.^[19] When platelets are exposed to high shear stress, they release particles expressing membrane receptors and cytoplasmic constituents termed as platelet microparticles (PMPs). PMPs have a direct involvement in malignant cell proliferation and growth. They also have an inherent ability to induce chemotaxis of many hematopoietic cells and increase their adhesive affinity to fibrinogen.⁽²⁰⁾ Survival of tumor cells in the blood stream is essential for metastasis. The majority of tumor cells do not survive in the hostile microenvironment after intravasation. Natural killer cells, cytotoxic lymphocytes capable of inducing tumor cell lysis, exert the major threat to tumor cells in the blood stream. Platelets are suggested to serve as a physical guard for the tumor cells in the blood circulation, allowing protection against immune elimination.^[21] Platelets also contain over 30 important angiogenesis regulating proteins. They have been recognized as a major source of vascular endothelial growth factor (VEGF) in serum as the platelet pool comprises of more than 80% of total circulating VEGF in patients with cancer as well as healthy individuals.^[22] Platelets also contain proteins that inhibit angiogenesis including platelet factor-4 (PF-4) and endostatin.^[23] They have also been shown to uptake and store proteins that regulate angiogenesis.^[24] Evidence also suggests that platelets can be conditioned *in vivo* by tumor cells to deliver anti-angiogenic proteins.^[25] It has been known for almost a half a century that, during

hematogenous spread, tumor cells modulate the hemostasis of the host and interact with circulating host cells including platelets. It was recognized that cancer cells characterized by the potential of hematogenous dissemination are usually able to aggregate platelets.^[18]

Cancer patients frequently present with signs of thrombosis, and these are most severe if the disease has progressed to a metastatic stage.^[19] Severe forms of thrombosis include disseminated intravascular coagulation, migratory thrombophlebitis and pulmonary embolism, indicating aberrant platelet activation and aggregation.^[18] However, even if thrombotic events are not detected, coagulation parameters are often increased in cancer patients, and platelet turnover is generally enhanced.^[26,28] These clinical observations indicate a potential relationship between the blood coagulation system, platelet functions and cancer spreading via the bloodstream.^[15] According to Gurevitch, Nelken, Danon in 1948 who studied the morphology of platelets using sodium chloride solution, observed that platelets suspension in 0.85% NaCl solution had very few tiny spicules. When platelets were suspended in 0.60 % NaCl solution, spicules were seen on most of them. Platelets suspension in 0.40 and 0.34% NaCl solution had a few sword like protrusions.^[27] In the present study similar results were observed. Lissy k. Krishnan in 1998 studied normal and agonist treated platelets, before and after ADP treatment and observed their findings under phase contrast microscopy. The increase and decrease of agonist causes change in shape and clumping of platelets. When treated with ADP an increased extent of aggregation of platelets was seen compared to the control.^[29] In the present study platelets showed marked variation in size and numbers in precancerous lesions whereas for normal platelets using 0.34 % and 0.85 % NaCl solution most of the platelets were larger in size and spicules were observed on few of them. Eight patients with OSMF while two patients with leukoplakia showed an increase in platelet aggregate along with variation in size and number which correlated with the degree of dysplasia. However no explanation can be given as to why there was less platelet aggregation in the remaining cases. These variations could be attributed to the size of the sample and methods of assessment.

CONCLUSION:

In the present study, fragility of 15 samples of normal platelets and 15 samples of precancer lesion were examined in hypotonic NaCl solutions. All

these platelet samples showed identical behavior in different NaCl concentrations. Dissolution started at a concentration of about 0.40 % NaCl and was complete at a concentration of about 0.34% NaCl. Thus the present study provides baseline information on the morphologic features of platelets in the oral precancer subjects. It is likely that the study of platelets in oral precancer will have wider clinical implications in the very near future. Therefore, additional studies with a larger study population have to be conducted to ascertain these findings and to elucidate the issue.

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