International Journal of Current Advanced Research
ISSN: O: 2319-6475, ISSN: P: 2319 – 6505, Impact Factor: SJIF: 5.995
Available Online at www.journalijcar.org
Volume 6; Issue 7; July 2017; Page No. 4753-4757
DOI: http://dx.doi.org/10.24327/ijcar.2017.4757.0576

Research Article

INTERACTIONS OF LECTINS IN THE RED BLOOD CELLS OF ORAL SQUAMOUS CELL CARCINOMA PATIENTS: A COMPARATIVE STUDY

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ARTICLE INFO

Article History:

Received 20th April, 2017
Received in revised form 14th May, 2017
Accepted 18th June, 2017
Published online 28th July, 2017

Key words:

Hemagglutination, Glycoconjugates, Diagnosis

ABSTRACT

Background: With the progress of cancer research, a peculiar group of proteins has become the subject of great consideration, namely lectins. These proteins have the ability to bind reversibly to carbohydrates with high specificity. The rationale behind our study is to evaluate the hemagglutination reaction of lectins in blood samples of Oral squamous cell carcinoma (OSCC) and healthy individuals

Methods: Thirty different seed extracts reacting non-specifically with human RBC were selected for the present study. They were tested against the RBC of Oral Squamous cell carcinoma patients by Hemagglutination and inhibition test.

Results: Out of 30 lectins extracted from the seeds of various plants, ten lectins showed positive agglutination with both OSCC and normal subject's blood sample. Eight of which, agglutinated RBC of both OSCC patient and control group. On the contrary, two lectins extracted from PHA and Con A agglutinated the OSCC patient's RBC more than the normal blood samples and showed highly significant value(P value >.001) as compared to normal control

Conclusion: Through similar studies if their presence can be reliably ascertained, lectinology may help to define yet disclosed role for this class of proteins in early diagnosis of OSCC by acting as biomarker.

INTRODUCTION

Oral Squamous Cell Carcinoma represents a major public health concern, especially in view of the increasing incidence and prevalence rates observed along the last few years with a remarkable incidence of being the sixth most common cancer worldwide and a fairly onerous prognosis, encouraging further research on factors that might modify disease outcome.[1] In an attempt to overcome these difficulties and to better understand the process of malignancy, a unique group of proteins, namely the lectins, has become the subject of special attention. Lectins are proteins, chiefly containing covalently bound carbohydrates that bind distinctively to the saccharide moieties in glycoproteins on the cell surface without modifying them chemically.[2] They are divided into different groups based on their carbohydrate binding specificity including N-acetylglucosamine binding, fucose binding, galactose binding, glucose binding, mannose binding, N-galactosamine binding, sialic acid binding etc.

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Binding is reversible and all lectins have more than one specific carbohydrate binding site. They contain soluble factor that can agglutinate red blood cells.[3] It is important to comprehend that the agglutinins differ widely from each other, that they exhibit substantial specificity, and that different agglutinins should be regarded as distinct molecular species with differences and biological characteristics.

In some cases the agglutinating action is inhibited by specific sugars as Morgan and Watkins (1953)[4] had first observed and the receptor groups of the erythrocytes possess carbohydrate moieties. The inhibition of the agglutination is explained by the interaction of the sugars with the reactive site of the lectins in such a manner that they can no longer react with their specific receptor groups Krtipe (1956).[5] Selective lectin agglutination was first observed by Aub *et al* (1963)[6] Their application to diagnose various types of tumor cells has rapidly developed in cancer research. Bhalla and Roy[7] reported positive and negative specific plant agglutinins to differentiate leukemic and non-leukemic red cells. Recently, Sharan and Haline[8] have reported the use of lectins in recognition by cell surface molecules. These studies have revealed striking changes in lectins agglutinability and specificity to carbohydrates. A sirnple and noninvasive blood

based maker is required for early diagnosis as well as for monitoring progress of treatment. Hence hemagglutination test is employed in biological and biomedical research. Those lectins which can agglutinate red blood cells irrespective of any specific blood group are known as "Nonspecific haemagglutinins".[9] Cell biologists have made the most conspicuous use of lectins as probes in the studies of cell surface structure and function. To the best of author's knowledge no other study has been carried out on the lectins in comparing the agglutination activity in erythrocytes of normal subject and patients of OSCC. In the present study non-specific lectins have been used as tool to investigate differences between the same.

MATERIALS AND METHODS

The prospective cohort study was done in the Department of Oral Pathology & Microbiology and the blood samples were collected from Department of Oral Surgery, Department of Biotechnology, Jaipur Dental College, Government Dental College, Jaipur and OPD clinics of Bhagwan Mahaveer Cancer Hospital & Research Institute. All the samples were collected with the ethical approval of ethical committees of bhagwanmahaveer cancer hospital, Jaipur. Jaipur dental college, Jaipur, India. The research study was conducted on 40 histopathologically diagnosed OSCC blood samples of the patients with the age range of 35-75 years, with male to female ratio of 2.3:1. The blood samples were collected after attainment of written consent forms from the patients. Patients selected for this study were explained in detail about the lesions and all the necessary investigations were carried out. A formal informed written consent was taken from all the patients. Lastly, the blood of the patient was drawn by a 21 gauge needle under sterile conditions and the blood sample was collected in EDTA vials which were sent to the Department of Oral Pathology and Microbiology for the procedure. Blood samples were also collected from healthy volunteers whose age and sex were matched. Lectins were extracted and purified using the method given by Dunsford & Bowley[10]. The reaction of blood with semipurified lectin was carried out to investigate the haemagglutination reaction of blood in cases of OSCC and normal subjects. Thirty different seed extracts reacting non-specifically with human RBC were selected for the present study. They were tested against the RBC of Oral Squamous cell carcinoma patients by Hemagglutination and inhibition test.

Preparation of Lectins

Extracts were prepared following the technique of Dunsford and Bowley.[10] Seeds from laxmi beej bhandar, shop no:6, aara machin,jhotwara Jaipur, Rajasthan which were from different plants were taken and washed circumspectly with distilled water to confiscate all unwanted materials and dried completely with the help of tissue paper to remove all its moisture. The dried seeds were grounded to powdered form with the help of pestle and mortar and soaked in phosphate buffer saline (PBS) with a magnetic bead which was placed in the solution inside the flask and kept for 24 hours with incessant stirring on a magnetic stirrer. After stirring, the solution was filtered with cheese cloth in a beaker to obtain the pure lectin solution free from other proteins and impurities. The filtrates were centrifuged at 3000 rpm for sixty minutes and the supernatant was filtered through Whatman's filter paper No. 42 and stored in sterile ampoules at -4°C. Sodium azide (.01%) was added as preservative.

Dialysis Purification Method

The dialysis tubes were tied from one end and ethanol purified lectins were poured in it and tied from other end also. Then these dialysis tubes were dipped in 500 ml of phosphate buffered saline in a beaker and kept for 24 hours with cold stirring on a magnetic stirrer in a refrigerator. Then these mixtures were filled in Eppendorf tubes and centrifuged at 3000 rpm for 10-15 minutes. After centrifugation supernatant was taken out and preserved for further use.

Preparation of Red Cell Suspension

The blood samples drawn from OSCC patients and control were washed thrice with excess of PBS. The packed red cells were re-suspended in PBS to prepare a 5-10% of suspension of red cells. The suspension was poured in the micropipettes which were centrifuged at 3000 rpm for 5 minutes.

Haemagglutination Test

Frozen lectins were thawed at room temperature just before the experiment was to begin. A drop of erythrocyte suspension and equal amount of lectin were added in Eppendorf tubes. Appropriate control was run along with the test. The reaction was followed for 15-20 minutes visually with naked eyes. Microscopic examination was used to confirm the same. Thereby, agglutination activity of lectins was observed. The complete procedure was carried out under laminar air flow.

Inhibition Test

The sugar inhibition test was carried out as described by Bhatia and Boyd. 0.2M sugar solution was prepared for the HI test. One drop of lectin (titre 1:8) was mixed with equal volume of .2M sugar solution in a micro titre plate in different cavities and kept for 2 hours at room temperature (25-30°celsius). One drop of red cell suspension was added to the respective cavities and mixed well. The results were noted visually with naked eye or microscopically after 30 minutes.

Statistical Analysis

The two by two or (fourfold) contingency table was applied on the ten non specific lectins which represents two sets of observations. Paired results were analysed using Chi-square_2 by 2. A p value >0.05 was considered to be statistically significant. The chi-square statistics were calculated from the Fischer's table test and the significance between the two variables was noted by the formula given below.

RESULTS

An array of 30 lectins was taken to examine against OSCC patient's blood of which 10 of them showed positive agglutination reaction. Other lectins were negative for both control and patients with OSCC. Results of the haemagglutination reaction to blood samples of OSCC patients and control are shown in Table 1. Phaseolus vulgaris agglutinated RBC's of OSCC patients in the highest percentage of 80% followed by Concanavalin with a percentage of 70% and they did not agglutinate the control blood samples. Lectins which showed agglutination in both control and OSCC were Vigna mungo (42.5% in OSCC and

Table 1 Results of Haemgglutination Test on Array of 30 Lectins

S.No	Semipurified lectins	OSCC positive	OSCC negative	Control positive	Control negative
1.	Maize (Zeamaiyes)	0	40	0	40
2.	Hara moong (Vigna radiate)	0	40	0	40
3.	Urad dal (Vigna mungo)	16	24	10	30
4.	Masoor Dal (Lens culinaris)	0	40	0	40
5.	Arhar Dal (Cajanuscajan)	13	27	7	33
6.	Cowpea (Vignasinensis)	0	40	0	40
7.	Chana dal (Cicer Arietinum)	17	23	15	25
8.	Flaxseed (Linumusitatissimum)	0	40	0	40
9.	Rice (OryzaSativa)	0	40	0	40
10.	Castor (Ricinuscommunis)	0	40	0	40
11.	Acacia (Acacia arabica)	0	40	0	40
12.	Coriander (Coriandrumsativum)	0	40	0	40
13.	Cumin (Nigella sativa)	0	40	0	40
14.	Onion seeds (Allium cepa)	0	40	0	40
15	Black pepper (Piper nigrum)	0	40	0	40
16	White pepper (Piper nigrum)	0	40	0	40
17	Til (Seasamumindicum)	0	40	0	40
18	Gwar beans (Dolichas lab lab	0	40	0	40
19	Alfaalfa (Medicagosativa	0	40	0	40
20	Wheat (Triticumturgidum)	0	40	0	40
21	Gram (Lens Cutilaris)	0	40	0	40
22	Cucumber (Cucumismelo)	0	40	0	40
23	Papaya (Carica papaya)	0	40	0	40
24	Soyabean (Glycine Max)	14	26	6	32
25	Jack fruit (Artocarpusheterophyllus)	16	24	10	30
26	Tamarind (Tamarindus Indica)	14	26	11	31
27	Rajma (Phaseolus Vulgaris)	32	8	0	40
28	Pea (Pisum Sativam)	14	26	5	35
29	Peanut (Arachis Hypogea)	18	22	8	32
30	JackBeans (Concanavalin)	28	12	0	40

Table 2 Positive Agglutination Reactions

Botanical name of seeds showing positive agglutination with OSCC blood.	Percentage positivity in Control	Percentage negativity in Control	Percentage positivity in OSCC	Percentage negativity in OSCC
Phaseolus vulgaris	0%	100%	80%	20%
Concanavalin	0%	100%	70%	30%
Vigna mungo	25%	75%	42.5%	57.5%
Cajanuscajan	17.5%	82.5%	43%	57%
Cicerareitinum	37.5%	62.5%	42.5%	57.5%
Arachis hypogea	20%	80%	45%	55%
Pisum sativum	12.5%	87.5%	35%	65%
Tamarindus indica	27.5%	72.5%	35%	65%
Artocarpusheterophyllus	25%	75%	45%	60%
Glycine max	15%	85%	35%	65%

Table 3 P value of Seeds with Positive Agglutination Reaction

S.No	Lectins	% Positivity in OSCC	% Positivity in Control	P value
1	Vigna Mungo	42.50%	25%	.01
2	Cajanus Cajan	43%	17.5%	.001
3	CicerAreitinum	42.50%	37.5%	.05
4	Concanavalin	70%	0%	.0002
5	Arachis Hypogea	45%	20%	.0002
6	Pisum Sativum	35%	12.5%	.0002
7	Phaseolus Vulgaris	80%	0%	.001
8	Tamarindus Indica	35%	27.5%	.5
9	Artocarpus H.	45%	25%	.002
10	Glycine Max	35%	15%	.0001

25% in control), Cajanuscajan (32.5% in OSCC and 17.5% in control), Cicerareitinum (42.5% in OSCC and 37.5% in control), Arachis hypogea (45% in OSCC patients and 20% in control), Pisum sativum (35% in OSCC and 12.5% in control), Tamarindus indica (35% in OSCC patients and 27.5%% in control), Artocarpusheterophyllus (45% in OSCC patients and 25% in control) and Glycine max (35% in OSCC samples blood and 15% in control) as shown in Table 2.

We had two variables, positive agglutination with OSCC blood and positive agglutination with control blood samples. 40 histopathologically diagnosed OSCC were sampled as part of a study along with a control group. A contingency table was created to display the same. The p values were calculated and Fischer's test was applied to test for statistical significance. To put together, out of these ten purified lectins, eight of them demonstrate positive agglutination with both control and OSCC blood sample while in tandem *Concanavalin* and *Phaseolus vulgaris* demonstrate positivity for OSCC blood sample and did not agglutinate the control group, 80% positivity with OSCC blood samples was shown by Phaseolus Vulgaris whilst 70% positivity in OSCC samples by Concanavalin.

Further the p value test was useful which substantiate highly significant results for Concanavalin with p value >.0002 and Phaseolus vulgaris with a p value >.001in OSCC and control, the other lectins were Glycine max with p value>.0001, Pisum sativum .0002, Arachis hypogea .0002, Artrocarpus heterophyllus .002, cajanuscajan .001, vigna mungo .01, shows statistically significant p values while CicerAreitinum

and Tamarindus indica showed non-significant results with p values > .05 and 0.5 for the same as shown in Table 3**.**

Inference Of The Haemgglutination Inhibition Assay

Further, the Haemagglutination Inhibition (HI) reaction was carried which clearly differentiates by giving pronounced reactions.

- C+/T+ where both the samples (control + test) gave +/+, - HI reaction indicating that identical cell surface saccharide determinants shared by OSCC patient and normal subject. In this case there were eight lectins which gave this reaction, like; Pisum sativum, arachis hypogea, glycine max etc.
- C-/T+, where both the samples (control+ test) gave dissimilar reactions, they were -/+, HI + indicating that saccharide determinants in the red cell surface which bind a lectin are not identical. In this particular case two lectins, Phaseolus vulgaris and Concanavalin A out of thirty clearly suggest that there is difference in the red cell surface receptors of OSCC patient and normal subject.

DISCUSSION

Lectins are bound specifically to carbohydrate containing groups on the cell surface and biochemical or microscopic methods are employed to examine the consequences of such bindings. The cell surface carbohydrates are in most cases products of the cell, although in addition, they may comprise glycoproteins and glycolipids that have been secreted by other cells and then adsorbed onto the cell surface.[11] This leads to alteration of carbohydrates moieties of major erythrocyte antigens and any changes in the cell carbohydrates, during malignant development, involve the blood antigens. Tewarson *et al* (1993)[12] is of the opinion that all cell surface membrane components play a prominent role in neoplastic behaviour. The adhesiveness of surfaces depends upon the relative magnitudes of the attractive and repulsive forces between them. Repulsive forces can be arises from the electrostatic repulsion between charged surfaces and operate during considerable distances, in contrast attractive forces in general decrease more rapidly with increasing distance. Linkages between negatively charged groups such as carboxyl by bivalent ions such as calcium may also take place. Intercellular adhesions exhibit more complex behaviour. Cells such as erythrocytes, which are comparatively non adhesive both to substrate and to each other, carry a high negative surface charge.[13]

The Leguminosae family of plant lectins contain ions Mn^{+2} and Ca^{+2} which are associated with a series of highly conserved amino acids involved in carbohydrate binding processes which is in accord with the above explanation.[14] The lectins which demonstrate positive agglutination in our study were Vigna Mungo, Phaseolus vulgaris, Cajanus Cajan, Arachis hypogea, Concanavalin, CicerAreitinum, Tamarindus indica, Artrocarpus heterophyllus, Pisum sativumand Glycine Max. Lectins from *Vigna Mungo* were extracted, purified. It is an enzymatic lectin which has been and extensively characterized by Hankins and Shannon 1978.[15] On the contrary, in *Cicerareitinum* which has been known to show hemagglutination activity were isolated from *Cicerareitinum.* The lectins were found to be monomer with many sugar binding sites.[16] Lectin isolated from *Cicer arietinum* possessed carbohydrate specificity as well as antiproliferative activity towards oral cancer cell lines. In our study the hemagglutination was not statistically significant as p value >.5, as it showed positive agglutination reaction with both OSCC blood samples and with control.

On the other hand in ***Glycine max*** (Soyabean) the agglutinin was homogenous by chromatography on DEAE-cellulose, electrophoresis at various pH values Pallansch and Liener (1953).[11] Our results are in concordance with the results of Liener & Pallansch who found hemagglutination reaction in blood samples of OSCC patients by a large amount of lectins. The reason behind the hemagglutination of OSCC blood sample in present study could be because of high titre of lectins. It has been proved that malignant erythrocytes were agglutinated only by large amounts of the agglutinin, and normal blood cells showed agglutination even in small amounts.Magyarosy, E., Sebestypen, A (1952).[17] The two lectins from the seeds of Phaseolus Vulgaris and Concanavalin A showed agglutination with OSCC blood samples but did not agglutinate the blood of normal blood cells and exhibited negative haemagglutination inhibition assay which depicts that role of saccharide containing site is indicated by the fact that the specific but not other saccharide, haptens can inhibit agglutination by CON A or PHA.The evidence that lectins bind specific carbohydrate groups on surface membranes comes from the observation that when particular sugars are present in mixtures of blood cells with lectin, agglutination is inhibited.Similarly in our experiment, clumping of blood cells by Concanavalin A and Phaseolus vulgarisis blocked by the simple sugars glucose and mannose. Hence the distinction in the lectins based on the different activities of some plant agglutinins toward the normal and cancerous blood of humans is seen. Concanavalin A (CON A) binds specifically to alpha forms of mannose and glucose but is unreactive with beta forms Goldstein and Poretz, (1986)[18] Soon it was found that Con-A also agglutinated malignant cells. In the present study, the property of lectin agglutination in blood on normal and OSCC patients has been observed. Both CON A and PHA revealed strong agglutinating activity in the malignant RBC's while didn't show any reaction with normal blood which was further confirmed by the negative HI test. These results indicate that lectin array is useful for harnessing the differentially expressed glycoproteins between cancer and healthy control subjects. This supports the fact that use of lectins as a biomarker in cancer research can gain importance, probably as a result of its wide applicability, versatility, and reliability. Further analyses of lectins as biomarkers should be undertaken to improve our understanding of the processes. Changes of these enzymes in malignant transformation indicate that cell surface carbohydrates of RBC's shows alterations and these can be detected using lectinology. Thus its application is based on the expression of aberrant glycans on the surface of erythrocytes. The detection of such aberrant glycans by lectins is indicative for an early diagnosis.

CONCLUSIONS

Hence, knowledge about lectins is limited to certain plants or animal sources and further research is important to identify

lectins and their importance in as many sources as possible. There should be many lines of experimental investigation opening up for exploitation. In the search for their functions, the end may not be in sight but, at last, it is conceivably around the corner. Present study shows that Lectins could be the next generation medicines if efficient research is contributed in their understanding.

Acknowledgements

We acknowledge and thank all the non-authors who have helped us in the present study.

Declarations

- Ethics approval and consent to participate: The blood samples were collected from Department of Oral Surgery, Department of Biotechnology, Jaipur Dental College, Government Dental College, Jaipur, India and OPD clinics of Bhagwan Mahaveer Cancer Hospital & Research Institute, India, and The blood samples were collected after attainment of written consent forms from the patients. Patients selected for this study were explained in detail about the lesions and all the necessary investigations were carried out. A formal informed written consent was taken from all the patients
- Consent for publication: 'Not applicable'
- Availability of data and material: All data generated or analysed during this study are included in this published article
- Competing interests: None
- Funding: Jaipur Dental College, Government Dental College, Jaipur, India.
- Authors' contributions:FN:Collection of blood samples and analysis and tests, PA:Collection of blood and analysis, PG:Lectin samples collection, NS: contributor in writing the manuscript, SN:Preparation, analysis and tests.

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How to cite this article:

Nida Fahmi *et al* (2017) 'Interactions of lectins in the red blood cells of oral squamous cell carcinoma patients: a comparative study', *International Journal of Current Advanced Research*, 06(07), pp. 4753-4757.
DOI: http://dx.doi.org/10.24327/ijcar.2017.4757.0576

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