JKAU: Med. Sci., **Vol. 16** No. 4, pp: 49-60 (2009 A.D. / 1430 A.H.)

DOI: 10.4197/Med. 16-4.4

Granzyme B, Is It a Cause for Clinicopathological Paradox in Medullary Breast Carcinoma?

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Abstract. Medullary carcinoma is famous of lymphoid infiltrate and improved prognosis. Cytotoxic T lymphocyte with positive Granzyme B constitutes a major subset within the infiltrate. The aim was to study Granzyme B expression and medullary carcinoma behavior. Twenty six female patients were admitted at Dr Fakeeh and Al Hayat Hospitals in Jeddah, K.S.A. from March 2004 to January 2007. Sixteen patients were below 45 years and ten were above. Granzyme B was positive in 75% of the first group and 60% in the second group. Granzyme B expression was positive in 62.5% of infiltrating ductal carcinoma with *in situ* component while it was 80% without *in situ*. Granzyme B expressed in 100% of medullary carcinoma, and 60% in others ($p < 0.001$). 4 lymph nodes out of six were affected in the medullary carcinoma while in others it was 18 out of 20 with no significant statistical differences. Granzyme B+ve Cytotoxic T lymphocyte were 100% in medullary carcinoma while Granzyme B+ve tumor cell was 33.3%. The values were 60% and 20% in others. N0 showed 100% +ve Granzyme B, N1 showed 71.4% and N2 showed 50%. Granzyme B, in a way or another is responsible for damaging cancer cells, leading to lost ability to invade lymphatic vessels or to grow in regional lymph nodes.

Keywords: Granzyme B, Medullary, Breast carcinoma.

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Accepted for publication: 03 August 2009. Received: 18 April 2009.

Introduction

A number of clinical indices are expected to be related to cancer progression and to predict the prognosis of cancer patients. Among those indices the metastatic status of regional lymph nodes was thought to be one of the most significant candidates as a prognostic factor. Some other factors, *i.e*. tumor size, histological subtype of the tumor, and magnitudes of tumor infiltrating lymphocytes are also considered to be important[1]. The cancer progression and patient prognosis are dependent upon both, the biological characteristics of the cancer cells and the immunological status of the host. In reference to biological characteristics, p 53 mutation, over expression of epidermal growth factor (EGF) receptor, HER-2/neu and many other molecular events in cancer cells have been reported to be involved[2]. Regarding the host immune response, the number of tumor infiltrating lymphocytes and dendritic cells, or the production of immunoglobulin directed against the cancer cells, are likely to affect cancer progression as well as the patient survival[3].

Granzyme B (GRB) is a major component of cytoplasmic granules of cytotoxic T-lymphocytes and natural killer cells. In cellular immune reactions, GRB was produced by activated cytotoxic lymphocytes and initially stored in cytoplasmic granules[4]. Cytotoxic T lymphocytes (CTLs) lyses their targets by means of two distinct mechanisms involving granule exocytosis or the cross linking of so-called death receptors on the target cells. The first mechanism depends on the actions of several constituents of the secreted granules that contain the pore forming molecule, perforin together with a variety of granule associated enzymes. Of these enzymes, GRB is the main effector when dealing with target cell apoptosis. After secretion by the CTLs, GRB binds to the mannos-6-phosphate receptor on target cells and then enters the target cell through receptor mediate endocytosis. Subsequently, GRB is released from these endosomes into the cytoplasm of the target cell because of the pore forming capacity of perforin. Subsequently, GRB induces cell death by activating the caspases[5]. The second cytotoxic mechanism exerted by CTLs occurs through a subset of the tumor necrosis factor receptor superfamily, called the death receptors. Death receptors (FAS) cross linking induces a caspase cascade that will lead to the destruction of the target cell[6].

The primary mechanism by which GRB initiates cell death is activation of the caspases through caspase-10. However, under circumstances where caspase-10 is absent or dysfunctional, GRB can act through secondary mechanism including activation of the other caspases and direct cell killing by cleavage of non caspase substrates. The redundant functions of GRB ensure the effectiveness of granule mediated cell killing. Even in target cells that lack the expression or function by mutation of one or more of the caspases, providing the host with overlapping safeguards against aberrantly replicating, non self cells [7]. GRB can enter target cells by autonomously crossing the cell membrane, or via a receptor mediated pathway, and the co-existence of perforin facilitates intracellular trafficking of GRB in the target cells[8]. Overall, the net effect of caspase activation is to halt cell cycle progression, disable homeostatic and repair mechanisms; initiate the detachment of the cell from its surrounding tissue structures; disassemble structural components, and mark the dying cell for engulfment by surrounding cells and macrophages[9].

Medullary carcinoma (MC) of the breast is a unique subtype of infiltrating ductal carcinoma (IDC) that is characterized by a prominent lymphoid infiltrate, a syncytial growth pattern, and anaplastic morphology[1,2]. Patients with MC have better prognosis than those with other subtypes of IDC. This is a biological paradox, since its clinical behavior is not in agreement with its poorly differentiated histology and high proliferation rate. Recently reported, that MCs are infiltrated by significantly greater numbers of GRB-positive activated cytotoxic T-lymphocytes (CTLs) than poorly differentiated ductal carcinoma. CTLs destroy target cells by inducing programmed cell death or apoptosis. CTL-triggered apoptosis is mediated by two major pathways: through the release of cytotoxic granules, such as perforin and granzyme, thus resulting from the Fas / Fas ligand / receptor interaction[6].

Patients and Methods

This retrospective study was conducted at Dr. Soliman Fakeeh Hospital and Al Hayat Hospital in Jeddah, K.S.A. from March 2004 to January 2007. Twenty-six patients were enrolled in this study; all were females with breast mass. They were presented in the out-patient department complaining of breast mass, none of those masses were discovered through any medical organized program. All masses were

discovered by the patient. All patients were subjected to a routine pre-operative assessment with control of any co-morbid disease, especially in the older patients. The initial procedures were lumpectomy or frozen section. The definitive procedure was modified radical mastectomy, this was done to the twenty six patients enrolled in the study. All specimens were examined macroscopically to determine absence or presence of other mass or masses, and to identify lymph nodes. Microscopic examination with hematoxylin and eosin stains for histological typing (ductal, lobular, or terminal ductal lobular units). To examine if it was accompanied by *in situ* components, and if it was invasive or totally *in situ* grading of the mass, then determine the lymph node status to detect any nodal deposits as it was essential to classify tumors as medullary and other subtypes.

Granzyme B detection was done using monoclonal mouse anti-human GRB (clone GRB-7), DAKO code No. M-7235 which qualitatively detects the presence or absence of GRB. The antibody can be used for labeling paraffin embedded tissue sections fixed in formalin. Heat induced epitope retrieval in a 10 mmol/L. Tris buffer / 1 mmol/L EDTA, pH 9.0 is recommended. Thin tissue sections should not dry out during the treatment or during the following immunohistochemical staining procedure. Cells labeled by the antibody display cytoplasmic staining pattern corresponding to the granular localization of the antigen[10].

Statistical Analysis

Statistical analysis was done using Fisher's test to obtain Z-value, Chi-square used to compare the distribution of a categorical variable, and from the standardized table, the degree of probability was obtained [11].

Results

Table 1 shows the relation between GRB expression and the age of the patients included in the study, there were 12 (75%) patients with positive expression in patients under 45 years, and 6 (60%) in the age group above 45 years. This was statistically not significant ($p > 0.5$).

Table 1. GRB expression to age.

Age	No.	GRB +ve	GRB –ve	Statistics
< 45 years	16	12 (75%)	4 (25%)	$X^2 = 0.24$
> 45 years	10	6 (60%)	4 (40%)	P > 0.5 NS

Tissue diagnosis showed that GRB (Table 2) was positive in 10 cases (62.5%) out of 16 with tissue diagnosis of IDC accompanied by *in situ* component, with more expression (80%) when the diagnosis was IDC without *in situ* component. The comparison between positive cases for GRB in both IDC + *in situ* and purely IDC was not significant statistically ($p > 0.5$).

Table 2. Relation of histopathology to GRB expression.

Diagnosis	No.	GRB +ve	GRB –ve	Statistics
IDC + *In situ*	16	10 (62.5%)	6 (37.5%)	$X^2 = 0.88$
IDC	10	8 (80%)	2 (20%)	$P > 0.5$ NS

Regarding tumor subtyping, six medullary and twenty other subtypes were obtained. All the cases showed lymphocytic infiltrate of variable intensities, in which the most intense was seen in the medullary cases. GRB was expressed by a 100% in the medullary type and 60% in other subtypes. This difference ($Z = 3.6$) was highly significant ($p < .001$) (Table 3).

Table 3. Relation of tumor subtype to GRB.

Subtype	No.	GRB +ve	GRB -ve	Statistics
Medullary	6	6 (100%)	-	$Z = 3.6$
Other subtypes	20	12 (60%)	8 (40%)	$p < .001$ HS

Regarding the percentage of distribution of +ve GRB in CTLs (Fig. 1 and 2), it was 69.2% in all the tumors, 60% in other subtypes and it was 100% in medullary type. The differences between medullary and other subtypes was highly significant ($Z = 3.6 – p < .001$) Table 4.

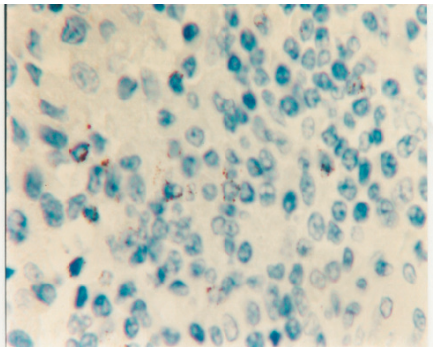

Fig. 1. GRB in CTLs.

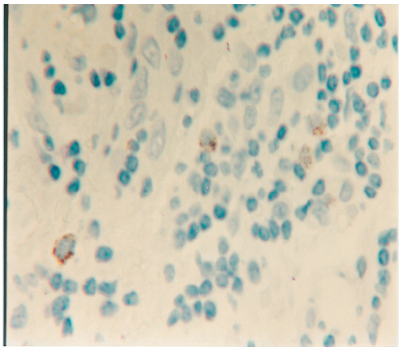

Fig. 2. GRB in CTLs.

Table 4. Distribution of GRB positivity in CTLs.

Type	Cases	CTL +ve Gr-B	Statistics
Medullary	6	6 (100%)	Z = 3.6
Other subtypes	20	12 (60%)	p < .001 HS
All	26	18 (69.2%)	

Regarding the positive tumor cells for GRB (Fig. 3 and 4), it was 23% in all tumors; it was 20% in other subtypes while it was 33.3% in medullary tumors. The difference in distribution between medullary and others was not significant ($p > 0.5$) (Table 5).

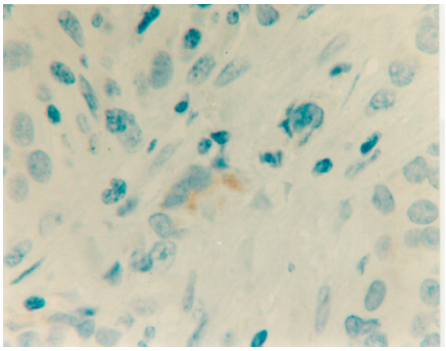

Fig. 3. GRB in tumoral cells.

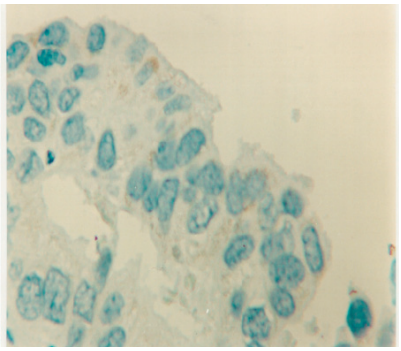

Fig. 4. GRB in tumoral cells.

Table 5. GRB distribution in tumor cells.

Type	Cases	Tumor Cell +ve GRB	Statistics
Medullary	6	2 (33.3%)	$X^2 = 0.4$
Other subtypes	20	4 (20%)	p > 0.5 NS
All	26	6 (23%)	

Comparing tumor GRB positivity and L.N. status showed that N0 associated with 100% positivity, N1 associated with 71.4% positivity while N2 showed 50% positivity (Table 6).

Table 6. Relation of the L.N. status to Gr-B.

L.N.	No.	GRB +ve
N0	4	4 (100%)
N1	14	10 (71.4%)
N2	8	4 (50%)

There were no significant differences ($p < 0.5$) in –ve L.N. in medullary (2/6) and those among other tumors (2/20) (Table 7).

Table 7. L.N. status *vs.* histological type.

Type	Case	-ve N	+ve N	Statistics
Medullary	6	2	4	$X^2 = 1.87$
Others	20	2	18	$p < 0.5$ NS

Discussion

Medullary carcinoma is a morphologically and biologically distinct subtype that, despite exhibiting cytologic anaplastic features, it has a more favorable prognosis than other subtypes at similar stages of differentiation. This is at least in part due to the presence of a prominent lympho-plasmacytic cell infiltrate. Analysis by mass spectrometry revealed that the antigen targeted by the dominant clones in the oligoclonal B cells was not a cancer specific antigen, but the cytoskeletal protein B-actin. MCB exhibits an increased rate of apoptosis and apoptotic MCB cells were shown to expose actin on the cell surface, permitting its recognition by the humoral immune system[13].

The primary mechanism by which GRB initiates cell death is activation of the caspases through caspase 10. However, under circumstances where caspase 10 is absent or dysfunctional, GRB can act through secondary mechanism including activation of other caspases and direct cell killing by cleavage of non caspase substrates. The redundant functions of GRB ensure the effectiveness of granule mediated cell killing, even in target cells that lack the expression or function of one or more of the caspases[12].

Regarding the age the expression, they were more in the younger age group (< 45) as it was 75%, while it was 60% in the older age group (> 45). However, it was no significant statistically this may present a more potent immunological response in the younger age group.

The relation between histopathology and positive GRB expression showed that when the tumor was totally IDC, it was associated with more positive GRB expression with less positive expression, then when it was associated with *in situ* component. Nevertheless, this difference was not statistically significant. This finding may go well with the recent observation stating that cells of

intraductal component may be more aggressive than the invasive component as it was proved it may be more HER-2-neu positive.

GRB positivity was expressed in 69.2% of our patients; this expression was detected on CTLs. Among the tumor cells GRB positivity was detected in six cases (23%). All those six cases who presented GRB positivity were in the tumor masses that were also positive for CTLs. However, none of those six cases had positive GRB in tumor tissue with negative GRB in CTLs. Now, it was determined, that the main population of cells stained with the antibody was in the tumor infiltrating lymphocytes rather than in the tumor cells. This finding was supported by Hu *et al.*[4] who stated that in the majority of cases, all tumor cells were stained negatively for GRB Only 16% of his cases stained positively for GRB, while 100% of his cases stained positively for GRB in CTLs. On the contrary, in the work of Kontani *et al.*[3] the main population of cells that were stained with the antibodies were cancer cells in the tumor tissues rather than immune cells infiltrating into the tumors. This can be explained by possible different phases of immunological response progression.

In the current work medullary breast cancer, it showed 100% positive GRB expression in CTLs and 33.3% GRB expression in tumor cells. On the other hand, other subtypes showed 60% and 20% respectively. Statistically, there was a highly significant difference among the GRB positivity in CTLs with no significant difference among GRB positive tumor cells. This granzyme positivity in CTLS can be explained in part; a key mechanism controlling the good prognosis for this type of tumor and solve the pathological paradox of medullary carcinoma[14]. Agreed with this explanation about the reason for an improved outcome of medullary carcinoma, as in the present study, the percent for +ve GRB was 100%.

In vivo studies showed that intravenous administration of immune GRB, gene-modified lymphocytes led to the suppression of HER2-over expressing tumor growth, and prolonged animal survival because of continuous secretion of immunoGRB molecules into blood and lymph fluid. These results demonstrated that the chimeric immune GRB molecule, which was capable of antibody-directed targeting and GRB-mediated killing, has therapeutic potential against HER2 tumors,

especially in cases in which caspase-dependent apoptosis was inhibited[15].

Despite the presence of these proteins close to their sites of action in the cancer cells from an early stage, the cancer cells containing these proteins did not appear to be damaged or lysed microscopically. This suggests that the cytotoxic granules produced by CTILs plays a role in suppressing metastasis of cancer cells to regional lymph nodes, rather than killing cancer cells adjacent to the immune effector cells.

Regarding expression of GRB in breast tissue, it was found to be 100% in patient with N0, 71.4% in N1 and 50% in N2. It is clear that the expression of GRB was more in N0 and whenever there was less GRB expression there were more lymph nodes affection. Kontani *et al.*[3] who had 61% +ve GRB, found that N0 showed 79% expression while it was 40% in N1 and only 25% in N2, going hand in hand with ours. Practically lymph node affection was more than three times greater in other subtypes, as –ve L.N. was 33% in medullary cancer compared to 10% in other subtypes. This finding also supports the previous explanation for the clinical paradox of medullary breast carcinoma.

Conclusions

Granzyme B in a way or another is responsible for the damage of cancer cells, leading to lost ability to invade lymphatic vessels or to grow in regional lymph nodes. It can be concluded, that there are more than one clone of CTLs; one responsible for inhibition of metastasis, the other is responsible for killing tumoral cells.

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هل جرانزيم ب سبب فى التضارب الإكلينيكى الباثولوجى فى سرطان الثدى النخاعى؟

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المستخلص. يتميز سرطان الثدى النخاعي بانتشار ليمفاوي وعواقب متحسنة. الخلايا الليمفاوية السامة ت التى تحتوى على الجرانزيم ب تمثل المجموعة الكبرى فى الانتشار. الهدف هو دراسة العلاقة بينه وبين جرانزيم ب. تمت دراسة ٢٦ حالة بمستشفى فقيه والحياة جدة بالسعودية في الفترة من مارس ٢٠٠٤ إلى يناير ٢٠٠٧م، ١٦ أقل و١٠ أكثر من ٤٥ سنة. جرانزيم ب كان إيجابيا في ٧٥٪ من المجموعة الأولى، وفي ٦٠٪ من المجموعة الثانية. جرانزيم ب كان إيجابيا في ٦٢,٥٪ من المنتشر المصاحب لمكون موضعي، و٨٠٪ من المنتشر فقط. كان جرانزيم ب إيجابيا في ١٠٠٪ من السرطان النخاعي، ومصاحب لتأثر غدي ليمفاوي أقل، و٦٠٪ من الحالات الأخرى (هام جداً إحصائيا).

كان يوجد ٤ غدد إيجابية الورم من ٦ في النخاعي، والأخرى كان ١٨ من ٢٠، وهذا غير هام إحصائيا. جرانزيم ب كان إيجابيا في الخلايا الليمفاوية في ١٠٠٪ في السرطان النخاعي،

و٣٣,٣٪ فقط داخل الخلايا الورمية. كانت النسبة ٦٠٪ و٢٠٪ في الأخرى. كان جرانزيم ب إيجابيا في١٠٠٪ في ن، و٧١,٤٪ في ن١، و٥٠٪ في ن٢. جرانزيم ب بطريقة أو بأخرى فإنه مسئول عن إحداث الضرر بالخلايا، مما يؤدى إلى عدم قدرتها على اختراق الأوعية الليمفاوية أو النمو في الغدد الليمفاوية.