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The Anti-Tumor Activities of Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) and Recombinant Fas Molecules in Breast Cancer Tumor Microenvironment

Seham Abou Shousha[1], Malak Zoheir[2] , Mahmoud Hemida[3], Yasmine Shahine[4]

[1]Departement of immunology & Allergy, Medical Research Institute, Alexandria University, Egypt
[2]Departement Pathology, Medical research institute, Alexandria university , Egypt
[3]Departement Surgery, Medical research institute, Alexandria university , Egypt
[4]Departement of Microbiology & Immunology, Faculty of Pharmacy & Drug Manufacturing, Pharos University in Alexandria, Egypt

Email: yasmine.shahine@pua.edu.eg

ABSTRACT

Background: Breast cancer, the most common malignancy in women, is a major health problem that is characterized by a defect in cell death (apoptosis). TRAIL and Fas are members of TNF super family that can induce apoptosis and kill malignant cells as wells as bacterial and viral infected cells through activating both apoptotic extrinsic and intrinsic apoptotic pathways. As evasion of apoptosis is thought to be a mechanism of tumor escape, we aimed at the current study to investigate the apoptotic activities of TRAIL and recombinant Fas molecules in breast cancer tumor microenvironment.
Methods: Brest tumor/normal tissue samples were collected from 30 radically mestectomized breast cancer patients and cultured individually in absence and presence of either TRAIL or rFas molecules. Apoptosis level was measured immune-histochemicaly according to caspase3 staining reaction.
Results: Our results revealed that there is a significant increase in the level of induced apoptosis within the breast tumor tissue cultured with recombinant TRAIL molecules (TT) than that induced in those cultured with recombinant Fas molecules (TF) or in absence of either (Mean ranks 2.8, 2.2 and 1.1 respectively, $p<0.0001$). No significant increase in induced apoptosis levels observed within the normal tissue culture systems N, NF and NT (mean rank 1.8,2 and 2.2 respectively, $p = 0.396$) while there was a *significant* higher levels of induced apoptosis observed in the treated tumor culture systems than that in corresponding normal ones (TF vs. NF and TT vs. NT, $P<0.001$).
Conclusion: TRAIL and Fas have a significant value in selective induction of apoptosis for breast cancer cells within the TME with the superiority of recombinant TRAIL.

Key words: Breast cancer, tumor microenvironment, Apoptosis, TRAIL, Fas.

INTRODUCTION

Breast cancer is the most common cancer in females with nearly 1,5 million new cases worldwide and half million related deaths.[1] Normal breast development is controlled by a balance between cell proliferation and apoptosis where there is strong evidence that tumor growth is not just a result of uncontrolled proliferation but also of reduced apoptosis. Apoptosis is a very tightly programmed cell death with distinct biochemical and genetic pathways that play a critical role in the development and homeostasis[2]

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Apoptosis pathways are well organized and strictly regulated cell death mechanisms, the two well-known apoptotic pathways are the mitochondrial pathway and extrinsic pathway. The extrinsic pathway is characterized by the ligation of the death ligands to their death receptors. Death receptors mainly belongs to the TNF super family such as TNF, Fas and TNF-related apoptosis inducing ligand (TRAIL) receptors. These death receptors have an extracellular domain to engage the ligands and an intracellular death domain (DD) responsible for transmitting the death signal from the surface to the intracellular signaling pathways. The activation of Fas or TRAIL receptors DR4 and DR5 leads to receptor clustering and intracellular recruitment of proteins into a death-inducing signaling complex (DISC), activating caspase 8 or caspase 10 which in turn activates the executioner caspase 3 leading DNA fragmentation and the late apoptosis events.[3]

TRAIL is a type II transmemberane protein that can induce apoptosis and kill malignant cells as wells as bacterial and viral infected cells through activating both apoptotic extrinsic and intrinsic pathways. TRAIL has been also reported to be an inducer of tumor cell apoptosis, where its ability to cause toxicity in various cancer cells while leaving normal cells unharmed made it an exciting and potential cancer therapy. Four human receptors specific for TRAIL have been identified: the death receptors DR4 and DR5, and the putative decoy receptors DCR1 and DCR2 [4].

Fas, a cell surface protein that belongs to the TNF receptor family, can mediate apoptosis when bound to its natural ligand, Fas L. It is expressed in the thymus, liver, heart, and kidney. Fas L is predominantly expressed in activated T lymphocytes and NK cells[5] , where Fas / Fas L is a direct major pathway that cytotoxic T lymphocytes use to eliminate viral infected or transformed cells.[6]

Evasion of apoptosis is one of the most important hallmarks of cancer where apoptosis resistance has a key role in tumorigenisis and treatment resistance. There are different mechanisms implicated in the evasion of apoptosis by tumor cells, including impaired death receptor signaling**,** disrupted balance of pro-apoptotic and anti-apoptotic proteins **,** reduced caspase function, mutations in p53 and increased the expression of inhibitors of apoptosis proteins[7]. It has been demonstrated as well that expression of Fas L by apoptosis-resistant tumor cells enables a powerful 'counterattack' against antitumor immune effector cells, such as cytotoxic T cells, many of which are themselves sensitive to Fas L-mediated apoptosis.[8]

So we aimed at investigating the apoptotic activities of recombinant Fas molecules compared to well established pro-apoptotic activities of recombinant TRAIL in breast cancer tumor microenvironment (TME).

PATIENTS AND METHODS

Patients:-

A total of 30 Egyptian females who were scheduled for modified radical mastectomy for histologically proved breast cancer, were recruited from Department of Surgery, Medical Research Institute, University of Alexandria. They were all subjected to full history taking and clinical examination with special reference to the stage of the disease as well as lymph node involvement. The descriptive analysis of the clinico-pathological parameters of the subjects is illustrated in table-1.

Table-1. The clinico-pathological data of the patients enrolled in the study

Clinico-pathological Parameter	No	%	Min - Max
Age			
<50	12	40	Min. - Max.: 34 – 72
≥50	18	60	Mean ± SD: 52.2 ± 10.97
Histopathologic type			
Invasive Ductal Carcinoma	26	86.7	
Mucinious carcinoma	2	6.665	
Invasive lobular carcinoma	2	6.665	
Histologic grade			
I	2	6.67	
II	28	93.33	
Vascular invasion			
Negative	4	13.3	
Positive	26	86.7	
Tumor size			
≤2 cm	8	26.7	Min. - Max. 1.5 – 7.5
>2 – 5 cm	18	60	Mean ± SD: 3.67 ± 2.01
>5 cm	4	13.3	
L.N invasion			
Negative L.N.	6	20	Min. - Max. 0-11
Positive L.N.	24	80	Mean ± SD: 4 ± 4.17
Hormonal status			
ER			
-ve	2	6.67	
+	2	6,67	
++	14	46.67	

+++	12	40	
PR			
-ve	2	6.67	
+	6	20	
++	12	40	
+++	10	33.33	

Methods:-

Breast tumor tissue culture preparation:

Fresh sterile tissue from primary breast tumor, at least 2 cm thick, was obtained immediately after surgical resection. Each tumor sample was divided into two parts; one part for the routine histo-pathological studies and the other part was maintained in organ transportation medium on ice until used for the tissue culture.

Each tumor tissue sample was submerged in complete RPMI medium and equal volumes of breast tumor tissues were incubated at 37^{o}c in a constant atmosphere of 5% CO2 for 24 hours in absence and presence of appropriate concentration of recombinant TRAIL (10 ng/ml + 0.1 ng enhancer (=100:1) or recombinant Fas molecules (10 µl of 50 ng/ml). The same procedure was performed with breast normal tissue of the same excised breast. By the end of incubation period, cultured tumor and normal tissue were fixed in 10% phosphate-buffered formalin PH 7.4 for 24 hours then processed for preparation of microscopic slides for immunohistochemically detection of apoptosis. [9]

Assessment of apoptosis:

It was achieved by immunohistochemical semi-quantitative measurement of Caspase-3 staining reaction using a rabbit polyclonal IgG Lab Vision (Caspase-3 (CPP 32) Ab-4) as a primary antibody and a labeled streptavidin-biotin immunoenzymatic antigen detection system, (Ultra Vision Detection System, AntiPolyvalent, HRP/DAB Catalog #: TP-015-HD) according to the manufacturers' manual. The DAB chromogen yielded brown color reaction end point at the site of target antigen. Visual evaluation of immuno-staining was performed with a light microscope using a semi-quantitative scale according to the intensity of nuclear staining as follows: (-ve) No staining, (+) Weak staining, (++) Intermediate staining and (+++) Strong staining.[9]

Statistical analysis

All data were presented as mean and SD they were compared with the tabulated P value at the 0.05 level using SPSS statistical package (SPSS Inc., Chicago, IL). The following statistical tests were used: Mann-Whitney Rank-Sum test and Friedman rank test.

RESULTS

Apoptosis levels in breast normal and tumor tissue culture systems:

The distribution of apoptosis level according to caspase3 staining intensity is summarized in (Table-2) by number and percent.

Statistical analysis of the results revealed that, within the breast normal tissue culture systems, the levels of apoptosis detected in treated breast normal tissues either with recombinant TRAIL molecules or recombinant FAS molecules was insignificantly higher than those of untreated ones (mean ranks 2.2 , 2 and 1.8 respectively, P=0.396). (Table 2) (Fig. 1-3).

On the other hand, the level of induced apoptosis within the breast tumor culture system treated with recombinant TRAIL molecules was significantly higher than that of treated with recombinant Fas molecules or those of untreated ones (mean ranks 2.8,2.2 and 1.1 respectively, p < 0.001) (Table-2).

In addition, the levels of induced apoptosis within the breast tissue culture systems treated with either recombinant FAS molecules or recombinant TRAIL molecules were higher in the treated breast tumor tissue cultures than their corresponding treated normal ones (p<0.001) (Table-3).

Table-1. The level of apoptosis within the breast normal and tumor tissue culture systems.

Apoptosis Levels	N		NF		NT		T		TF		TT	
	No	%	No	%	No	%	No	%	No	%	No	%
-ve	28	93.33	26	86.67	24	80	26	86.67	0	0	0	0
+	2	6.67	4	13.33	6	20	4	13.33	12	40	2	6.67
++	0	0	0	0	0	0	0	0	13	43.33	10	33.33
+++	0	0	0	0	0	0	0	0	5	16.67	18	60
Mean Rank*	1.8		2		2.2		1.1		2.2		2.8	
P value**	0.396						<0.001					

*: Mean Rank value of groups of Friedman test **: Statistically significant at p ≤ 0.05

Table-2. Comparison between apoptosis levels in breast tumor tissue culture systems and breast normal tissue ones

Apoptosis Levels	T		N		TF		NF		TT		NT	
	No	%	No	%	No	%	No	%	No	%	No	%
-ve	26	93.33	28	86.67	0	0	26	86.67	0	0	24	80
+	4	6.67	2	13.33	12	40	4	13.33	2	6.67	6	20
++	0	0	0	0	13	43.33	0	0	10	33.33	0	0
+++	0	0	0	0	5	16.67	0	0	18	60	0	0
p value*	0.65				<0.0001				<0.0001			

*: p value for Mann Whitney -U test **: Statistically significant at p ≤ 0.05
T: Breast tumor tissue culture alone **TF:** Breast tumor tissue culture enriched with recombinant Fas molecules
TT: Breast tumor tissue culture enriched with recombinant TRAIL molecules
N: Breast normal tissue culture alone **NF:** Breast normal tissue culture enriched with recombinant Fas molecules
NT: Breast normal tissue culture enriched with recombinant TRAIL molecules

Figure-1. Untreated breast tumor tissue showing caspase 3 negative staining (IHC X100)

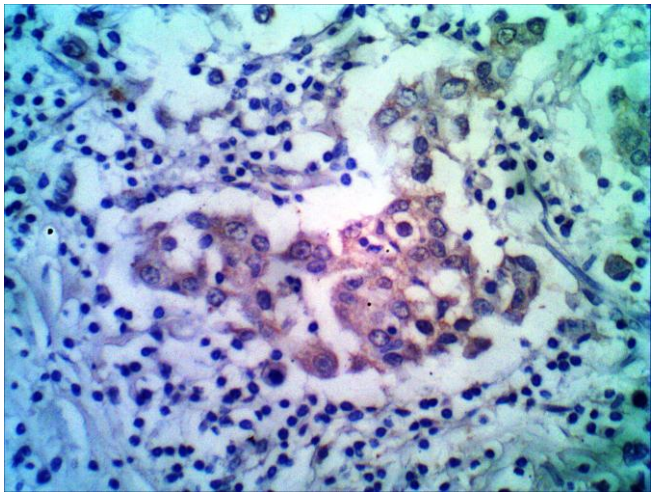


Figure-2. Breast tumor tissue enriched with recombinant Fas molecules showing moderate caspase 3 staining (IHC X100)

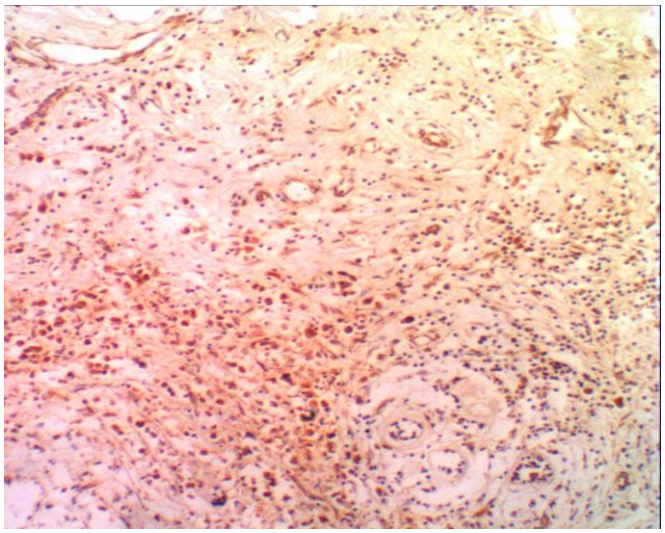
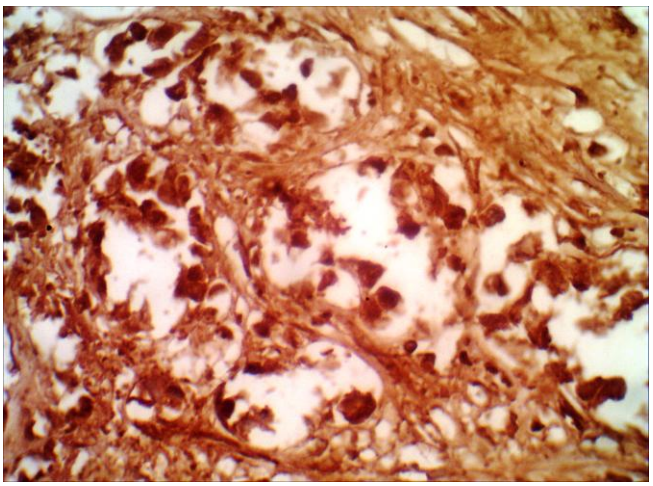


Figure-3. Breast tumor tissue treated with recombinant showing strong positivity staining (+++) of caspase-3 (IHC X100)



DISCUSSION

Manipulating the immune system or manipulating the TME to establish a tumor rejecting environment is now one of the most important strategies for fighting cancer. In addition, evasion of apoptosis or its resistance plays a vital role in carcinogenesis, which makes apoptosis a promising target for cancer immunotherapy.

The significant increased level of induced apoptosis in the breast tumor tissues cultured with recombinant TRAIL molecules in the current study than that cultured with recombinant Fas molecules and that cultured alone emphasis the important role of TRAIL and FAS in apoptosis induction.

The vast majority of studies explored the role of Fas and TRAIL pro-apoptotic activity as the primary endpoint of their stimulation. Many studies have explored the *in vitro* and *in vivo* anti-tumor activities of TRAIL, where, recombinant TRAIL was shown to have the ability to induce apoptosis in multiple malignant cell lines, either alone or in combination with various chemotherapy

agents or radiation via the activation of both extrinsic and intrinsic pathways [10].

Despite of the conventional breast cancer cell lines used by many researchers, these cell lines couldn't represent the heterogeneity of the TME and didn't demonstrate the actual interaction between TRAIL or FAS and tumor cells in the natural TME, taking in consideration the wide range of cells, mediators, effector molecules and different microenvironment constituents which can enhance or suppress the apoptotic effect of TRAIL and FAS. The designed tissue culture system used in the study enabled us to investigate the tumor tissue response to TRAIL and FAS induced apoptosis as well as cell-cell interaction in as nearly as actual TME.

A wide range of cells and cytokines naturally found in the TME can enhance or suppress the apoptotic effect of TRAIL and vice versa, i.e. TRAIL can influence the immune function of many cells in the TME. For example, gamma interferon (IFN-γ) produced by activated T cells and natural killer cells can synergistically induce apoptosis. It was also found that IFN-γ can enhance apoptosis induction by facilitating caspase 8 activation[11]. In addition, Diao et al (2013) elucidated that local TRAIL treatment decreased the number of regulatory T cells (Tregs) within the TME , where it was showed that TRAIL had the ability to induce apoptosis of the Tregs within the TME, but not of $CD4^+$ T cells and increased the number and enhanced the activity of tumor-specific $CD8^+$ CTL which indicates the important role of TRAIL apoptosis induction within the TME.[12]

Concerning Fas, apoptosis induction through Fas /Fas L pathway was the most established activity of Fas and Fas L and documented in many publications. [6] It is revealed that FasL is one of only a few molecules that immune cells use to kill cancer cells through apoptosis. Apoptosis induction as a cancer cell targeting strategy is mainly accomplished by tumor-infiltrating lymphocytes expressing Fas L as one its tumor surveillance mechanisms. [13] However, it was demonstrated that most cancer cells were shown to be resistant to apoptosis induction. In addition, stimulation of Fas could never be used therapeutically because of major side effects accompanied with FasL therapy such as massive apoptosis induction in the liver. [14]

In addition, several studies indicate that there are other outcomes of Fas receptor engagement by FasL other than apoptosis such as facilitating cell motility and invasion, induction of cell proliferation and metastasis.[15] where it was demonstrated that FasL expression by tumor cells is a associated with poor prognosis as it was found to have a role in tumor progression and invasion[16] as well as enabling a tumor counter attack on the activated T cells within the TME[17]. So blocking the Fas L expression within the TME by its enrichment with recombinant Fas molecules enabled the activated T lymphocytes and other effectors molecules within the TME to induce apoptosis to tumor cells and protects them from the tumor counter attack from one side and blocking the Fas L tumor promoting properties from the other side.

The non-significant increase in induced apoptosis levels observed within the normal tissue culture systems and the *significant* higher levels of induced apoptosis in the treated tumor tissue culture systems than that in corresponding normal ones can be denoted to the safety and selectivity of recombinant TRAIL and Fas in apoptosis induction in the tumor cells sparing the normal cells unharmed.

The selectivity of TRAIL is well established in many studies which make TRAIL a promising anti-cancer therapeutic strategy. Despite intensive investigations, little is known in regards to the mechanisms underlying TRAIL selectivity and efficiency. This may be due to the complexity of the interaction of TRAIL with its receptors; of which only two (DR4 and DR5) are death receptors while the binding of TRAIL with the decoy receptors DCR1 and DCR2 fails to induce apoptosis, thus over expression of the TRAIL decoy receptors, or downregulation of apoptosis inducing receptors can contribute to the TRAIL selectivity. [18]

Tuettenberg et al (2012) demonstrated that the administration of APG101, a glycosylated fusion protein consisting of the extracellular domain of human Fas and the Fc domain of human was considered safe and well tolerated in healthy volunteers. [19]. The selectivity of Fas can also be explained by the fact that the recombinant Fas molecules didn't in fact induce apoptosis but they only protect and facilitate the role of the effector immune cells within the TME.

CONCLUSION

According to our study findings, we can conclude that recombinant TRAIL and Fas have a significant value in selective induction of apoptosis for breast cancer cells within the TME with the superiority of recombinant TRAIL.

Conflict of Interests

Authors declare that there is no conflict of interests regarding the publication of this paper.

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