

## **Studies on the Mechanism of Action of Tumor Infiltrating Lymphocytes (TILs) Propagated Ex-Vivo**

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### **ABSTRACT**

**Background:** Tumor-infiltrating lymphocytes (TILs) play a key role in the immunogenic reaction against tumors. Although TILs are present in the tumor and recognize the antigenic characteristics of malignant cells, they are incapable of preventing cancer growth. However, pre-clinical data in vitro showed that activated TILs have the ability to kill autologous tumor cells. As a result, a number of clinical reinfusion trials using interleukin-2 (IL-2) - activated TILs have been conducted in the past few years.

**Purpose:** The therapeutic efficiency of TILs is not clearly understood, as only few studies have been performed on a limited number of patients and therefore this work aims at studying the mode of action of TILs propagated Ex vivo through investigating protease enzyme system using SDS-PAGE of gelatin zymography technique.

**Material and methods:** In this work, TILs as well as peripheral blood lymphocytes (PBL) were separated from patients suffering from breast cancer. Primary culture of TILs, PL as well as tumor cells were performed. A co-cultivation of TILs and tumor cells in the presence of IL-2 was done. Gelatin zymographic analysis using polyacrylamide gel electrophoresis of the protease enzyme system was performed.

**Results:** The work done revealed that: aggregation of TILs around tumor cells was clearly observed when a mixed culture of both TILs and tumor cells was co-cultivated in vitro. At a molecular level, there were two separate clear and sharp bands at approximately 72 and 92 kDa seen in gelatin zymography. The bands were much more clear with an extra band with dimeric structure above 92 kDa in cell lysate of the mixed culture of both TILs with tumor cells as well as their conditioned media.

**Conclusion:** The study findings may shed some light on a good possibility of synthesis and secretion of specific protease enzyme system for the lytic activity of TILs against tumor cells. Further work is strongly recommended for identification of the extra band produced due to the TILs and tumor cells interaction in addition to the possible specific novel therapy of TILs in cancer management.

**Key Words:** *Tumor infiltrating lymphocytes (TILs) - Cell culture - Breast cancer - Matrix metalloproteinases (MMPs).*

### **INTRODUCTION**

Tumor infiltrating lymphocytes (TILs) are lymphoid cells that infiltrate solid tumors, and that can be grown by culturing single cell suspension from tumors in the presence of interleukin-2 (IL-2) [11].

TILs play a role in the immunogenic reaction against tumors [14]. Although TILs are present in the tumor and recognize the antigenic characteristics of malignant cells, they are incapable of preventing cancer growth. However, pre-clinical data showed that TILs can kill autologous tumor cells ex-vivo [13,14]. The administration of gene modified TILs into patients with advanced cancer showed that these lymphoid cells can survive at the site of the tumor and in the circulation for several months [10]. The therapeutic effectiveness of reinfused TILs is not clearly understood as well as its mode of action.

Matrix metalloproteinases (MMPs) are a group of enzymes thought to be responsible for both normal connective tissue remodeling and accelerated breakdown associated with tumor development [5]. It has been shown that MMP-9 mediates cleavage of IL-2 R and down regulates the proliferative capability of cancer-encountered T-cells [12].

The present work represents a trial to separate TILs from tumor specimens obtained from patients with breast cancer so as to study the mechanism of action of TILs. In order to achieve this goal the work is performed for the detection of protease enzyme systems via gelatin zymographic analysis by SDS-PAGE technique.

## PATIENTS AND METHODS

Tumor tissues obtained from five patients suffering from breast cancer were taken during tumor excision in surgical department at the National Cancer Institute of Egypt (NCI), Cairo University.

### *Clinical data:*

Patients were diagnosed according to protocols adopted at Surgical and Medical Oncology Dept., NCI, Cairo University. The patients were subjected to the following clinical and laboratory investigations such as liver function tests, kidney function tests, chest x-ray, abdominal pelvic ultrasound (US), CT scanning and isotope bone scanning.

The clinicopathological features of the cases were

- CT chest was free.
- Bone scan was free.
- Abdominal US was free.
- All had invasive ductal carcinoma.
- All had grade II, LN -ve with only one case LN positive.

### *Reagents and chemicals:*

RPMI Tissue culture media, Foetal bovine serum (FBS) and antibiotics were purchased from Sigma and Ficoll separating solution Seromed® from Biochrom KG, Leonoremsr, Berlin.

### *Preparation of tumor infiltrating lymphocytes (TILs):*

TILs were separated according to a protocol provided by Cochet et al., 1998 [4] including the following procedure:

- 1- Freshly excised tumor tissue was washed twice with RPMI 1640 medium
- 2- The tumor tissue was minced into 3-5 mm pieces with a sharp scalpel.
- 3- The preparation was mixed with RPMI medium containing deoxyribonuclease (3600 units), collagenase (50 µg) and hyaluronidase (125 units) from 1-2 hrs at 37°C in rotating shakers.
- 4- The resulting cell suspension was filtered through a mesh with capacity 1 meq/gm to separate tumor cells from TILs.
- 5- RPMI 1640 medium was used for wash-

ing. The cells were resuspended at 3 - 5 x 10<sup>5</sup> cells per ml in RPMI 1640 medium supplemented with human recombinant IL-2 (200 units/ml).

6- The cells were transferred to 75 cm<sup>2</sup> tissue culture flasks and incubated at 37°C in a 5% CO<sub>2</sub> incubator. The medium was replaced twice a week.

7- Separation of peripheral mononuclear cells (PBMC) was done using Ficoll Hypaque according to Cochet et al., (1998), [4]. The same experiment performed with TILs was repeated with PBL. PBMC were adjusted to 10<sup>5</sup> cells/ml in RPMI 1640 and divided into two aliquots, one part was incubated with IL-2 as TILs rich fraction, the other was used as control without IL-2.

8- Tumor cells were kept in culture without IL-2 and co-cultivated with activated TILs. A control was set up by co-culturing tumor cells with non-activated TILs (IL-2- free).

9- SDS-PAGE for gelatin zymography was performed according to Birkedal-Hansen et al., (1982), [2]. The gels, (10%), containing 100 mg gelatin (Sigma Co. Type B from Bovine) were pre-electrophoresed for 30 minutes to minimize the loss of activity during electrophoresis by removing unreacted monomers and persulfate. Equal volumes of loading buffer and samples were mixed in microfuge tubes. 20µl of the last mixture were applied to each well. The standard enzyme was applied in one well in each run. The electrophoresis was done at 10mA till the samples run through the stacking gel then the current was raised to 20mA for 2 hours. The gels were washed in washing buffer: 50mM Tris HCl, (5mM CaCl<sub>2</sub>, 1µM ZnCl<sub>2</sub> and 2.5% Triton X-100, pH 7.5). The gels were stained in Coomassie brilliant blue and destained in destain I solution (50% methanol, 10% acetic acid and 40% distilled water), destain II solution (5% methanol, 7% acetic acid and 88% distilled water).

## RESULTS

Fig. (1), shows low cell density of tumor cells, since they were derived from primary culture of the tumor specimen from breast cancer patient. Fig. (2) shows gelatin zymographic pattern of protease by SDS- PAGE. The zymogram reveals the presence of matrix metalloproteinase MMP-2 and MMP- 9 according to their

molecular weights (72 kDa and 92 kDa respectively). The figure shows two clear bands of the protease enzyme system in case of cell lysate of all different cell types with differences in their activities. However, lane no. 4 reveals three sharp bands and the third one showed a dimeric structure. These were shown in the lysate of the mixed cell culture population of tumor cells with TILs in the presence of IL-2.

Fig. (3) shows the same previous bands of the protease enzyme system in conditioned medium obtained from different cell types, again, lane no. 4 shows an extra band with dimeric structure that represents protease activities in conditioned media from the mixed cell culture of tumor cells with TILs in the presence of IL-2.

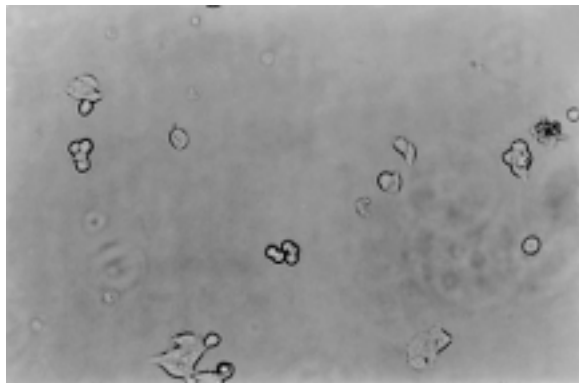


Fig. (1): Tumor cells obtained from the primary culture of tumor specimen from the breast cancer patient.

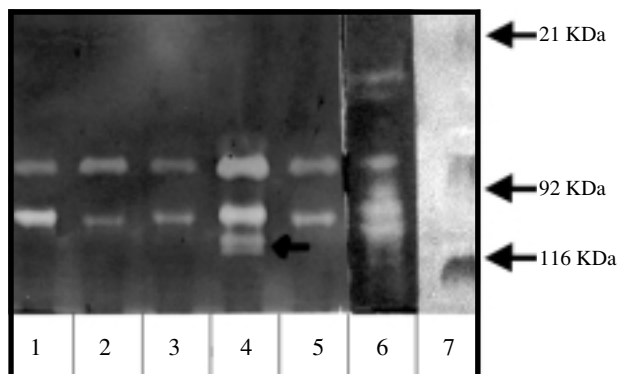


Fig. (2): Gelatin zymographic pattern of SDS-PAGE for protease activities from different types of cell lysates.

- Lane 1: Peripheral lymphoid cell (PLC).
- Lane 2: TILs1 obtained from filtered cells+PLC with IL-2.
- Lane 3: TILs2 obtained from packed cells with IL-2.
- Lane 4: Tumor cells in co-cultivation with TILs in the presence of IL-2.
- Lane 5: Tumor cells alone.
- Lane 6: Standard collagenase.
- Lane 7: Protein marker (21-116 kDa).

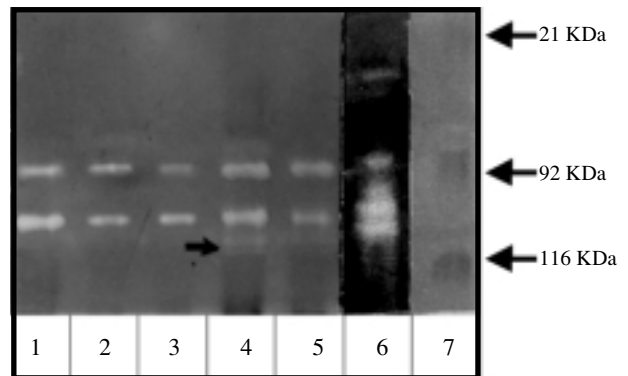


Fig. (3): Gelatin zymographic pattern of SDS-PAGE for protease activities from different types of conditioned media.

- Lane 1: Conditioned medium from PLC.
- Lane 2: Conditioned medium from TILs1.
- Lane 3: Conditioned medium from TILs2.
- Lane 4: Conditioned medium from tumor cells + TILs.
- Lane 5: Conditioned medium from tumor cells alone.
- Lane 6: Standard collagenase.
- Lane 7: Protein marker (21-116 kDa).

## DISCUSSION

Proteolytic degeneration of extracellular matrix is necessary for cell migration which is essential in physiological and pathological processes [6,7]. Two families of proteases, the plasminogen activator/plasmin system of serine proteases [2,3] and matrix metalloproteinases (MMPs) have been described [4,6]. In a previous study, it has been shown that rat IL-2 activated natural killer cells (NK) produce MMP-2 and MMP-9 [8]. Another study showed that human NK cells express and produce MMP-2, MMP-9 and MT1- MMP. These cells degrade the extracellular matrix through the secretion of MMPs [8]. In the present study, another type of lymphoid cells namely TILs was separated from patients with breast cancer. The work offers a trial for TILs mode of action via the secretion of MMPs. The work agreed with what have been reported by Abertsson et al., [1] who showed that MMPs represent a mechanism of action of human NK cells. It seems likely that both types of lymphoid cells (NK and TILs) act through the same mode of action for their tumor lytic activities. Furthermore, the present work revealed the morphological observation of the different cell types mainly peripheral blood lymphocytes cells (PBL), TILs and tumor cells. The figure list gave rise to an important observation that TILs have the ability to invade the tumor cells which was found to be destructed

via the production of MMPs released by TILs. The present work also elucidated the different activities of TILs in term of the bands shown by gelatin zymographic analysis according to their situation i. e. high activity of TILs shown in case of the co-cultivation with tumor cells particularly in the cell lysate of both cells. Further work is strongly recommended to identify which one is highly responsible for the activity of the proteases shown by gelatin zymography. However, it seems likely that cell-cell interaction could produce another type of protease above 92 kDa shown by the extra band with a dimeric structure previously mentioned. This type of protease could be responsible for the specific autologous lytic activity of TILs against tumor cells. This finding is similar to what have been previously reported for human NK cells which produce MMPs to allow for triggering their cytolytic potential [9].

In conclusion, TILs could be propagated *ex vivo* to produce MMPs in their media and this highlights the possibility of using MMPs released from TILs as a mechanism of action for their functionality as specific autologous lytic activity against tumor cells. Further work is strongly recommended to investigate the possibility of using TILs as a novel immunotherapeutic modality for the treatment of drug-resistant and radio-resistant types of cancer.

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