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May Mast Cells Have Any Effect in New Modalities of Cancer Treatment?

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1. Introduction

Multifunctional mast cells (MC) have been recently reported as effectors in the human innate and even adaptive immune system, besides their known roles in allergic disorders. First in vivo observations in the 1950`s suggested their possible role as anti-tumor cells around certain solid tumors and questioned their interactions with tumor cells (Prior, 1953). Later, in vitro murine mast cell cytotoxicity (MCC) against murine tumor cells was described in 1981 (Henderson, 1981; Ghiara, 1985; Richards 1988). However, to the best of our knowledge, there is no reported data on in vitro human MCC against human tumor cells. Current in vivo observations and implications from human pathological specimens are also very controversial. Moreover, there is still difficulty in obtaining and maintaining human MCs in cultures since they have low expansion potential. These facts have hampered in vitro human MC studies up to the last decades of the 20th century. The recent use of methylcellulose media for in vitro human MC cultures increased the knowledge and data strongly supporting an increasing role of MC as effector elements of innate immunity (Leskinen, 2003; Marshall, 2004; Della Rovere, 2009; Özdemir, 2007, 2011).

Ambiguous and mounting evidence also indicates that MCs accumulate around tumors and could either promote or inhibit tumor growth, most likely depending on environmental conditions. Presently, believers in the inhibitory role of MCs assume them to be inhibitors of tumor development through their cytotoxic pro-necrolytic/-apoptotic granules (Wagelie-Steffen, 1998; Leskinen, 2003; Kataoka, 2004; Pardo, 2007; Heikkilä, 2008). In fact, the MC has been long believed to have natural cytotoxicity against murine TNF-α sensitive tumor cells in the long term incubations (>24h), by either TNF-α dependent or independent pathways. TNF-α independent pathways like cathepsin G, NO, serine proteases, peroxidases, H_2O_2 etc. were also assumed to contribute to MCC (Henderson, 1981; Ghiara, 1985; Richards 1988). Moreover, in vitro murine MCC has been well demonstrated against TNF-α-sensitive murine WEHI-164 and L929 tumor cells. Murine MCC seems to be different from natural killer (NK) cytotoxicity by means of acting in long term against unusual targets such as WEHI-164 and L929 with different mediators like peroxidases (Henderson, 1981; Ghiara, 1985; Richards 1988). Moreover, the last decade of research demonstrates that MC granules have pro-apoptotic characteristics (Wagelie-Steffen, 1998; Leskinen, 2003; Kataoka, 2004; Pardo, 2007; Heikkilä, 2008). Chymase was shown to induce apoptosis, and apoptotic

pathway mediators such as FasL and granzyme B expressions were detected in mice and cultured MCs; respectively (Wagelie-Steffen, 1998; Leskinen, 2003; Kataoka, 2004; Pardo, 2007; Heikkilä, 2008). Thus, except for perforin (Pardo, 2007), MCs indeed have been proven to have all components of short and long-term cell-mediated cytotoxicity, which consist of the secretory pathway (via soluble TNF-α, chymase, serine proteases granzyme-B/-H), and non-secretory pathway (the death receptors Fas L and membranous TNF-α) (Özdemir, 2006, 2007, 2011).

Nonetheless, some researchers still consider MC as an enhancer of tumor development through their angiogenic effects, causing invasiveness and metastasis of tumor tissue (Özdemir, 2006). Some MC mediators such as heparin, IL-8 and tryptase are known to be responsible for angiogenesis (Ribatti, 2000). Yet, neither these mediators are the only known elements responsible from neoangiogenesis, nor are MCs the only resource. MCs also have a vast array of mediators, some of which have promoting, and others inhibitory effects on angiogenesis besides malignancies (Özdemir, 2006). The same researchers consistently based their theories on pathological specimen observations, showing an association between increased MCD and the worst prognosis in some cancers such as endometrial cancer, leukemia as well as lymphomas (Ribatti, 2009; Molin, 2002). Our correspondences against this conviction have been well documented in recent literature (Özdemir, 2006).

As summarized above, in this chaotic literature environment, our aim in this study was to investigate human MCC against NK- and lymphokine activated killer (LAK)-sensitive/ resistant human leukemia-lymphoma cells in short and long term coincubations by our established flow cytometric (FCM) cytotoxicity methods (Özdemir, 2003, 2007, 2011).

2. Material and methods

2.1 Mast cell (effector) development in methylcellulose and maintenance in suspension culture

A colony forming unit (CFU) -Mast was produced in vitro by our modified method (Özdemir, 2007, 2011). From several discarded patient samples, human bone marrow (BM) mononuclear cells (≥5x104) were obtained and suspended in 0.3 ml Iscove's Modified Dulbecco's Medium (IMDM) containing %1 Fetal Bovine Serum (FBS) after Ficoll (Sigma, St. Louis, MO). Cells were put into 3 ml serum-free methylcellulose medium [MethoCult™ SFBIT H4236, StemCell Technologies, British Columbia, Canada], supplemented with 200 ng/ml of SCF, 50 ng/ml of IL-6 and 1ng/ml of IL-3 (only at the beginning). All cytokines were purchased from Biosource, Camarillo, CA. We inoculated 0.3 ml of the mixed medium in the 12-well plate with a 16 gauge blunt needle and placed into an incubator. Every two weeks, the cells were fed with 0.3 ml new medium including 100 ng/ml of SCF and 50 ng/ml of IL-6. Thirty or more cells were scored as CFU-Mast in situ on an inverted microscope after 4 weeks. Before culturing in suspension, MCs are retrieved from only CFU-Mast`s in the medium and dissolved with >2-fold volume of phosphate buffer solution including FBS %10 at 6th weeks. Cells are centrifuged at 250xG for 5 minutes. They are suspended and cultured in complete IMDM supplemented with 100 ng/ml of SCF, 50 ng/ml of IL-6 and 2% FBS in 25cm^2 flask up to 8th weeks. The culture was started with ≤10^4 cells/ml and accumulated up to ≤2x10^6 cells/ml. The suspension culture was hemi-depleted every week and supplemented with 100 ng/ml of SCF and 50 ng/ml of IL-6.

2.2 Staining for verification of effector mast cells

Verification of MCs was done by May-Grunwald-Giemsa, Wright-Giemsa, acid Toluidine Blue staining and immunophenotyping on FCM. In brief, a colony was lifted with Eppendorf micropipette and spun down at 600 rpm for 5 minutes in the 4th and 6th week. Viability was checked with a trypan blue exclusion test. Cells from colonies were stained with May-Grunwald-Giemsa and Wright-Giemsa for verification purposes (Fig.1A1-4). MCs were fixated with a Carnoy solution and incubated for 2 minutes with acid toluidine blue to confirm their tryptase content as well. Furthermore, MCs were immunophenotyped for all related markers in FCM at 4th-8th weeks (Fig.1B1-3, Table 1). All monoclonal antibodies (mAb) were purchased from Immunotech, Inc. (Westbrook, ME).

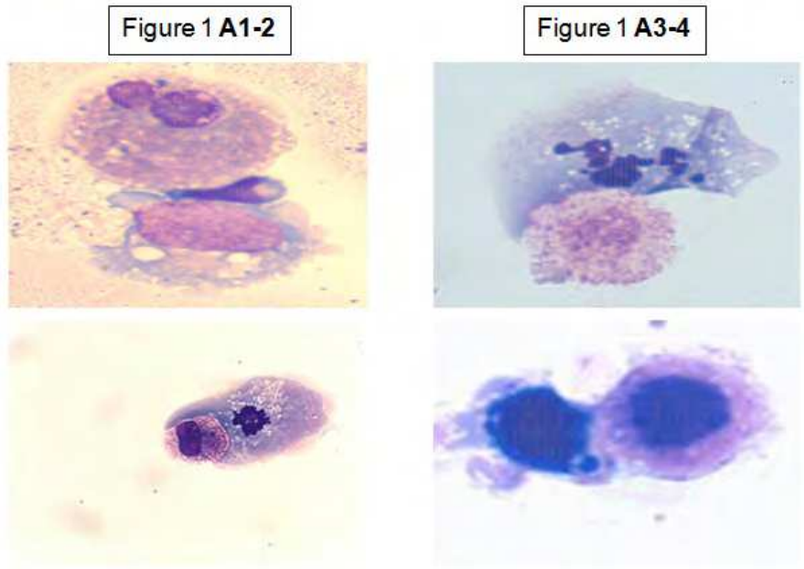

Fig. 1. A1- 4. Wright- Giemsa slides are showing conjugate formation between mast cell and both tumor cells (effector-target doublets). A1-2 show conjugate formation between mast and Daudi cells. A3-4 depict conjugate formation between mast and Raji cells.

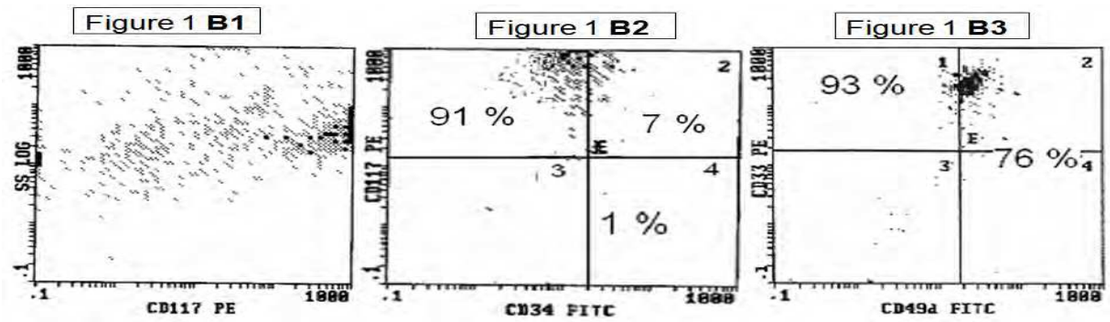

Fig. 1. B1- 3. Phenotyping of 4- week- old human bone marrow -derived mast cells on flow cytometry is shown in a representative sample. B1 shows CD117 (c-kit) expression vs. SS (granularity) of mast cells. B2 demonstrates ≥98% of cells already stained with CD117 and became CD34 negative. B3 illustrates 93% of cells stained with CD33 and 76% of cells were positive for CD49d. [The human effector (mast) cells produced from bone marrow were absolutely negative for CD19 in this study.]

Surface Marker Expressions	*4th-week*	*6th-week*	*8th-week*
CD14	0 %	0 %	0 %
CD15	93 %	3 %	2 %
CD19	0 %	0 %	0 %
CD33	93 %	25 %	19 %
CD34	7 %	1 %	1 %
CD38	0 %	0 %	0 %
CD44	93 %	85 %	84 %
CD45	0 %	0 %	0 %
CD49d	76 %	12 %	11 %
CD117 (c-kit)	91 %	95 %	98 %
HLA-DR	0 %	0 %	0 %

Table 1. The phenotypic characterizations of 4- to 8- week-old human bone marrow- derived mast cells by flow cytometry in representative samples are shown.

2.3 Target lymphoma and leukemia cells

The human malignant B-lymphoblastoid cell lines such as Daudi/ Raji and erythroleukemia cell line K562, known as "LAK- sensitive" and "reference cells" in cytotoxicity studies, were utilized in this study. In several experiments we also utilized LAK- resistant human acute myeloid leukemia (AML) cell lines (HL-60, DAMI and Meg-01) and discarded AML patient samples were used. All cell lines were obtained from ATCC (Manassas, VA) and maintained in RPMI 1640 culture media, supplemented with 10% FBS. Before coincubation, target and effector cell viability were determined by the trypan blue exclusion test; a viability of 90% was required to proceed.

2.4 Assessment of human MCC on FCM

Cytotoxicity was assessed by two different FCM cytotoxicity techniques utilizing DIOC18 or mAb staining for target cell labeling (Özdemir, 2007, 2011). The basic strategy of two-color FCM assay involves labeling target cells with a fluorescent membrane dye DIOC18, in addition to staining with PI, to identify dead cells. Alternatively, a new three-color FCM approach called as "flow cytometric mast cell-mediated cytotoxicity assay (FCM-MCMCA) " , which entails target cell marking with specific mAb (CD19) and with AnnV/PI colabeling to identify apoptotic/dead target cells, was used for some tumor cells. Briefly, we performed the following stepwise approach for analyzing the samples:

2.5 Pre-labeling target leukemia cells with DIOC18

A stock solution was prepared by dissolving DIOC18 (Sigma, St. Louis, MO) in DMSO (2 mg/ml) overnight with agitation. The target cells (10^6cells/ ml) were incubated with 10µg/ml of DIOC18 (final concentration) in 1 ml of PBS containing %3 FBS for 45 minutes at incubator. Then, target cells were washed with PBS three times to get rid of dye remnant. DIOC18 pre-labeling before coincubation was done for K562, Meg-01, HL-60, DAMI cells and the patient samples (Fig.1C1-E3).

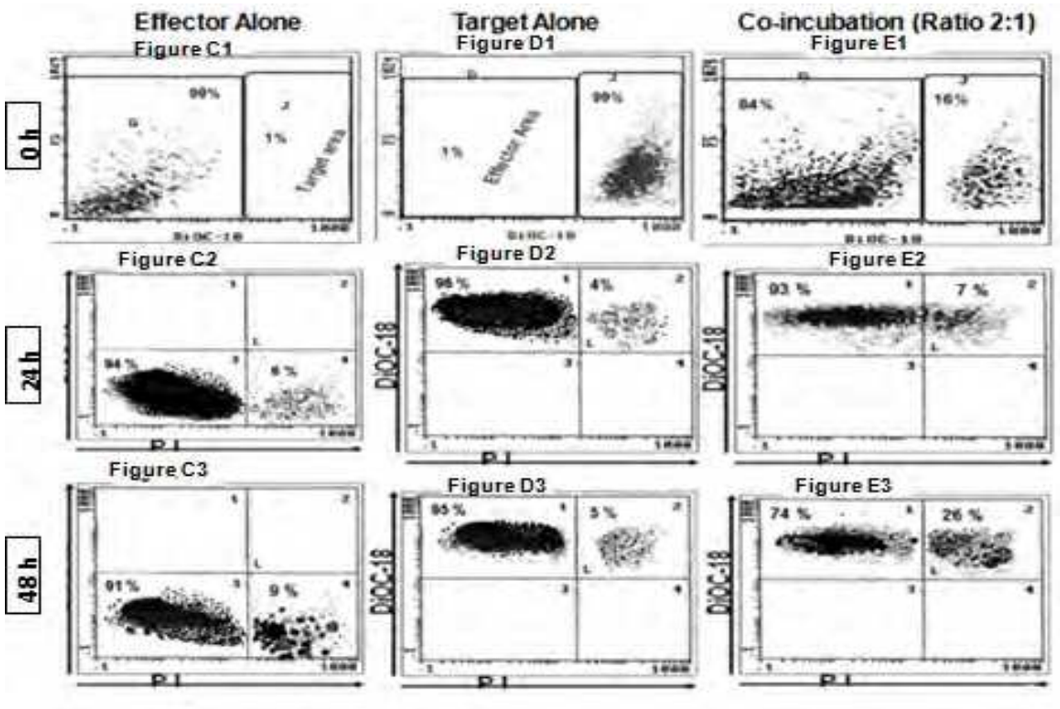

Fig. 1. C1- E3: Target cell (DAMI) death is shown by new flow cytometric assay at 24h and 48h in a representative sample. Identification of effector and target cells in alone/coincubation samples using target cell labeling (DIOC18) and cell size characteristics (FS) is shown in histograms at the first row. Spontaneous death and cytotoxicity evaluation of the cell populations alone or in co-incubation samples are shown with the rectilinear boxes of related histograms at the second and third rows. C1 shows location of effector-mast cell. Since effector cells are not pre-stained with DIOC18, they are DIOC18 negative and in a different area (Region G). C2 and C3 demonstrate the change in viability of effector cell alone population (DIOC18−/PI− viable cells: 94% and 91%; respectively) at 24h/48h. Spontaneous deaths in these effector alone samples are 6% and 9%; respectively. These histograms are obtained after gating on region G of a corresponding sample. D1 shows the target-DAMI cell population from target alone tube. As expected, target cells are very well-marked with DIOC18 (Region J). D2 and D3 depict the viability of the target population alone (DIOC18+/PI− viable cells: 96% and 95%; respectively) at 24h and 48h. Spontaneous deaths in these samples are 4% and 5%; respectively. These histograms are obtained after gating on region J of a corresponding sample. E1 obtained from an experiment where mast cells are co-incubated with DIOC18-positive DAMI cells at 2:1 effector/ target ratio. In this co-incubation sample; effector/target gatings are defined according to the target/effector alone (control) tubes. E2 depicts decrease in viability of previously gated target cells in J region of a corresponding co-incubation sample, 7% of them are stained with PI+ indicating necrotic killing (Region L2). In this representative sample, DAMI cell viability slightly decreased from 96% in the control to 93% after 24h co-incubation. The 4% of spontaneous death increased up to 7% with cytotoxicity mediated by mast cells during 24h co-incubation. E3 shows obvious decrease in viability of previously gated target DAMI cells, as 26% of them are dead. In this representative sample, DAMI cell viability further decreased from 95% to 74% throughout 48h co-incubation. The necrotic populations increased from a corresponding 5% of spontaneous death to 26% kill mediated by mast cell cytotoxicity in the co-incubated sample.

2.6 Coincubation

A few sets of human BM-derived MCs at 8th weeks of age, without any stimulation (PMA etc.) and human tumor targets were coincubated at certain effector/target ratios (1:1, 2:1, 4:1, 5:1) short-term and long-term. Human MCs were coincubated in vitro with human LAK-sensitive (K562, Daudi, Raji cells) and LAK- resistant tumor targets (Meg-01, HL-60, DAMI and patient cells) for the evaluation of human MCC. Tubes were centrifuged at 115xG for 5 minutes and incubated at 37°C in 5% CO_2 for short- (2h) and long-term (up to 48h) period. Although MCC is well-known to take place after long-term incubation (>18h), 2h and 48h coincubation times were selected so as not to miss any possible cytotoxicity. And these experiments were repeated several times with a few sets of MC colonies on different times. Samples were tested in duplicates for reproducibility and reliability.

2.7 Target lymphoma cell labeling with mAb and detection of death after coincubation

Labeling target cells were done by two different methods in this study. As mentioned earlier, the first one is DIOC18 prelabeling for leukemia samples, which is being done before coincubation. The second technique is based on marking the lymphoma targets such as Daudi and Raji cells after co-incubation with 10µl of mouse anti-human PE conjugated CD19 mAb (Immunotech, Westbrook, ME) for 20 minutes before staining for detection of death.
Detection of apoptosis/death in target cells in mAb labeling technique was done by staining with AnnexinV (AnnV) and propidium iodide (PI) (TACS™ AnnexinV-FITC; R&D Systems, Minneapolis, MN) for 20 minutes before FCM acquisition. AnnV+ and/or PI+ events were analyzed from gating on the events in the target population of the control as well as coincubation samples. Ann V+ events represent early apoptotic population, AnnV+/ PI+ dead (late apoptotic or necrotic) cells (Fig.1F1-I2). In the DIOC18 prelabeling method, counterstaining with the nuclear dye PI (R&D Systems, Minneapolis, MN) of target cells was also done for 20 minutes before acquisition to discriminate between live and dead target cells. PI+ events represent dead cell population in FCM (Fig.1C1-E3).

2.8 FCM acquisition and running

FCM was performed using an EPICS-XL MCL (multicarousel) (Coulter, Miami, FL) equipped with an argon laser (15 mW) source operating at 488 nm. The emission of three fluorochromes was recorded through specific band pass filters: 525 nm for FITC (FL1), 575 nm for PE (FL2), 620 nm for fluorospheres (FL3), and 675 nm for PI (FL4). The instrument was set for 4-color analysis. As the emission spectra of the three different dyes utilized in this bioassay interfere with one another, appropriate electronic compensations were adjusted by running individual cell populations stained with each dye consecutively through the EPICS. Once the compensations had been set, a gating was done on forward scatter (FS) (ordinate) versus log-scale fluorescence of mAb CD19 or DIOC18 (abscissa), to separate target cells from effector cells (Fig.1C1-I2). The FS gating was especially helpful for separating bigger DAMI and Meg-01 target cells from effector MCs. The log-scale fluorescence (CD19 or DIOC18) of abscissa was well enough to separate targets from effectors besides the aid of FS gatings. The different cell populations (target, effector and coincubated cells) were gated on with the help of isotype control samples. To measure target-cell death and apoptosis, CD19-/DIOC18- positive events were gated on, and analysis of log green fluorescence (AnnV) and/or log red fluorescence (PI) was performed depend on the cytotoxicity technique (Fig.1C1-I2).

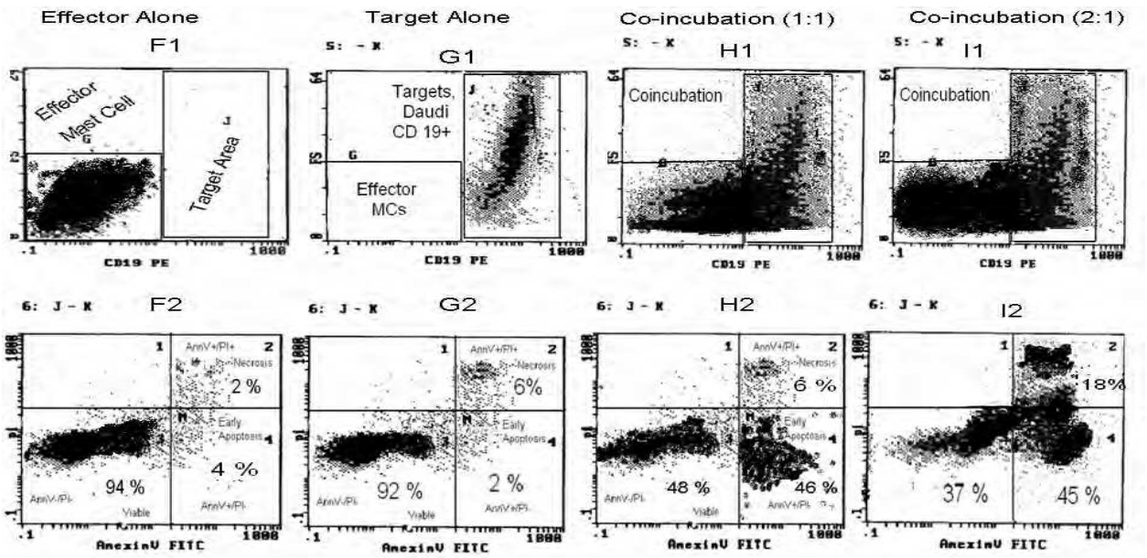

Fig. 1. F1- I2. Target (Daudi) cell kill, caused by mast cell- mediated cytotoxicity, is demonstrated by flow cytometric mast cell -mediated cytotoxicity assay (FCM-MCMCA) at 12h in a representative sample. Quadrant gating and determination of populations in fig. 1F1- H2 were defined by control samples consisting of target/effector alone tubes. The histograms in fig. 1G2-H2 were achieved from gating accordingly on target cell populations. 1F1 shows location of effector-mast cell in the histograms of FCM-MCMCA. Effector cells are seen as CD19 negative (effector alone sample). 1F2 demonstrates spontaneous kill in effector cell population in control tube. 1G1 shows target-tumor cells location. As expected, the target cell population is well-marked with CD19 in the histogram and there is no effector cell (target alone sample) in the tube. 1G2 shows spontaneous kill in the target cell population of the control sample. It shows 92% viable (Annexin-/ PI-) target (Daudi) cells with total 8% spontaneous killing including early apoptotic plus necrotic kill. 1H1 demonstrates co-incubated effector and target cells at 1:1 ratio after 12h (co-incubation sample). It reveals the changes in the target cell population in the quadrant J of the fig. 1G1 in coincubated samples after cytotoxic kill. Compared to fig. 1G1, decrease in size and amount of the target cell population of 1H1 indicates killing due to mast cell –mediated cytotoxicity. Effector cells in this sample are not stained with CD19 and they are in a different area. However; it is hard to say anything about conjugate formation between target and effector cells (effector-target doublets) in the co-incubation sample, and it seems probable that it is happening earlier. The doublets probably form and immediately dissolve during the beginning of this process. Thus, there is no reflection of conjugate formation in fig. 1H1. 1H2 histogram of co-incubation sample was acquired after gating on the population in the quadrant J of fig. 1H1 and reflects the alterations in that co-incubated target population after cytotoxic kill. It depicts obvious decrease in viability of the target cells from 92% to 48%. It shows increased death up to 52 %, which 6 % of killing was necrotic-late apoptotic (Annexin+/PI+) and 46 % of killing was early apoptotic (Annexin+/PI-). Obviously, decline in viability of the target cell population from 92% to 48% strongly reveals significant killing due to human mast cell- mediated cytotoxicity in vitro. Similarly, 1I1 shows further decrease in size and amount of the target cell population of 1G1 and 1H1 due to augmented cytotoxic kill at 2:1 ratio. And 1I2 demonstrates a decrease in viability of the target cell population from 92% to 37%, but death increased up to 53% at a higher ratio.

2.9 FCM data analysis

An average of 10,000 total events and 3,000 target cells were collected per sample. The gating for the target cells was based on the target-alone analysis and kept constant throughout all tubes to avoid exaggeration of the counts due to apoptotic body contamination. Cytotoxicity calculations were based on viable populations in target-alone (control) and co-culture (coincubated) tube analysis results. Viable target-cell percentage was determined, and calculations were based on the control-tube (target alone) values. Apoptotic and/or necrotic death happening in control tubes were identified as spontaneous, but those killed in coincubated tubes were identified as cytotoxic killing. We expressed the MCC as percentage based cytotoxicity:

2.10 Percentage based cytotoxicity (%, PC)

As mentioned, we defined MCC occurring in co-culture tube (coincubated sample) as percent (%, PC) cytotoxicity. And cytotoxicity calculations were based on viable populations in target-alone (control) and co-culture tube analysis results.

$$\text{Percent Cytotoxicity } (\text{PC}) = \frac{[\text{Control-viable cell\%}] - [\text{Coincubation-viable cell\%}]}{[\text{Control-viable cell\%}]}$$

Our in vitro experiments were repeated several times (n=27) with a few sets of MC colonies. Samples were tested in duplicates for reproducibility and reliability (mean coefficients of variation (CV) of 1.1% (95% CI, 0.8- 1.7%); r2=0.93, and p<0.001).

2.11 Comparison with chromium- ^{51}Cr release assay (CRA)

A standard assay was performed as previously described (Özdemir, 2007). Briefly, the assay is a standard 4h CRA using target cells that have been prelabeled with 100 µCi 51Cr (Perkin Elmer, Boston, MA) for 1h. Several concentrations of MCs were added to a fixed number of target cells (5,000) in a round bottom microtiter plate to a total volume of 0.2 ml. Following the 4h incubation, 0.1 ml of the supernatant was carefully harvested and counted on a scintillation counter (Packard, Downers Grove, IL). Maximum release was determined from wells including target cells and 10% sodium dodecyl sulfate in the medium. The PC was calculated using the following equation: (E–S)/(M–S)x100, where E is the experimental counts/minute, S is the spontaneous counts/minute and M is the maximum counts/minute. In the end, when we used some sets of our experimental data in comparing FCM cytotoxicity assays with CRA results, there was a significant correlation for PC (n=58; r= 0.95; P<0.001), similar to our earlier studies (Özdemir, 2007, 2011).

2.12 Cell staining with Wright/Giemsa for light microscopy

At the end of the coincubation period, the sample was gently mixed and 10µl was used to prepare slides at room temperature without cytospinning. The slides were then stained with Wright/Giemsa. Conjugate formations between some tumor cells and MCs were seen (Fig.1A1-A4).

2.13 Statistical analysis

Unless otherwise specified, all data are presented as means±SEM. The significance of differences between spontaneous and cytotoxic kill was determined using the paired

Student's t-test and/or Wilcoxon test. Correlations were estimated by Pearson correlation coefficients. Significance was considered as ≤0.05. All statistics were done using the Statistical Package for Social Sciences (SPSS-13 for Windows; Chicago, IL).

3. Results

This study demonstrated MCC against tumor cells by using human cells instead of murine effector/target cells. In vitro MCC against various tumor cells at different E:T ratios (1:1-5:1) was measured by FCM-MCMCA and DIOC18 methods on various periods (Table 2A-D).

Pecentage Based Mast Cell- Mediated Cytotoxicity (%, PC)				
Cell characteristics		**%, PC on Coincubation Times**		
LAK-sensitive	**Death Type**	**2h**	**18h**	**48h**
Effector (**Mast**) control/alone	Spontaneous	2±1	1±1	3±1
Target (**K562**) control	Spontaneous	3±1	5±1	5±1
Coincubation (5:1)	Cytotoxic	4±2	7±1	18±2
	N=	(3)	(9)	(9)
	P=	NS	NS	NS
LAK-resistant	**Death Type**	**2h**	**18h**	**48h**
Effector control /alone	Spontaneous	1±1	2±1	3±1
Target (**DAMI**) control	Spontaneous	2±1	5±1	7±1
Coincubation (5:1)	Cytotoxic	9±1	19±2	24±2
	N=	(3)	(9)	(9)
	P	NS	NS	NS
Effector control	Spontaneous	2±1	3±1	3±1
Target (**Meg-01**) control	Spontaneous	1±0	5±1	8±1
Coincubation (5:1)	Cytotoxic	7±1	20±2	27±4
	N=	(3)	(9)	(9)
	P=	NS	0.059	**0.031**
Effector control	Spontaneous	3±1	4±1	4±1
Target (**HL-60**) control	Spontaneous	5±1	8±1	11±2
Coincubation (5:1)	Cytotoxic	6±2	18±1	39±3
	N=	(3)	(9)	(9)
	P=	NS	0.050	**0.011**
Effector control	Spontaneous	1±1	3±1	4±1
Target (**Patients**`) control	Spontaneous	2±1	8±1	13±1
Coincubation (5:1)	Cytotoxic	5±1	20±1	22±2
	N=	(6)	(9)	(9)
	P=	NS	NS	NS

The death/killings (percentage based cytotoxicity) in this table reflect the death at a 5:1 ratio in this study. Spontaneous kill reflects the death in target or effector alone tubes. Cytotoxic death shows the target cell death by only mast cell- mediated cytotoxicity in coincubation sample. The percentage based cytotoxicity was calculated according to the given formula in the methods. All values are given as mean±SEM. P values reflect the significance of differences between spontaneous and mast cell mediated cytotoxic killing. N denotes the number of experiments were repeated in each series. NS means not significant.

Table 2A. In vitro human mast cell -mediated cytotoxicity measured by DIOC18 method against different human tumor cells is shown.

Percentage Based Human Mast Cell- Mediated Cytotoxicity (%, PC)				
Cell characteristics		**%, PC on Coincubation Times**		
LAK-sensitive	**Death Type**	**2h**	**12h**	**24h**
Effector (**Mast**) control	Spontaneous	3 ± 1	4 ± 1	5 ± 2
Target (**Daudi**) control	Spontaneous	2 ± 1	3 ± 1	6 ± 2
Coincubation (1:1)	Cytotoxic	21 ± 2	49 ± 2	53 ± 3
	N=	(9)	(9)	(9)
	P=	**0.048**	**0.009**	**0.005**
Effector control/alone	Spontaneous	4±1	3 ± 2	4 ± 2
Target (Daudi) control/alone	Spontaneous	4±1	3 ± 1	5 ± 2
Coincubation (2:1)	Cytotoxic	31±1	57± 2	63 ± 3
	N=	(3)	(9)	(9)
	P=	**0.039**	**0.001**	**0.001**
Effector control	Spontaneous	4±1	3 ± 2	4 ± 2
Target (Daudi) control	Spontaneous	2±1	4 ± 1	6 ± 2
Coincubation (4:1)	Cytotoxic	36±1	59± 2	67 ± 3
	N=	(9)	(9)	(9)
	P=	**0.030**	**0.001**	**0.001**
Effector control /alone	Spontaneous	2±1	3 ± 1	6 ± 1
Target (**Raji**) control /alone	Spontaneous	3±1	4 ± 1	8 ± 3
Coincubation (1:1)	Cytotoxic	13±1	16 ± 1	47± 3
	N=	(9)	(9)	(9)
	P=	**0.049**	**0.046**	**0.008**
Effector alone	Spontaneous	5±1	3 ± 1	4 ± 1
Target (Raji) alone	Spontaneous	7±1	5 ± 1	9 ± 3
Coincubation (2:1)	Cytotoxic	19±1	26 ± 1	67± 3
	N=	(3)	(9)	(9)
	P=	**0.044**	**0.016**	**0.001**
Effector alone	Spontaneous	4±1	6 ± 1	5 ± 1
Target (Raji) alone	Spontaneous	3±1	7 ± 1	8 ± 3
Coincubation (4:1)	Cytotoxic	19±1	30 ± 1	69± 3
	N=	(9)	(9)	(9)
	P=	**0.040**	**0.014**	**0.001**

The death/killings (percentage based cytotoxicity) in this table reflect the death at 1:1, 2:1, 4:1 ratios in this study. Spontaneous kill reflects the death in target or effector alone tubes. Cytotoxic death shows the target cell death by only mast cell-mediated cytotoxicity in the coincubation sample. The percentage based cytotoxicity was calculated according to the given formula in the methods. All values are given as mean±SEM. P values reflect the significance of differences between spontaneous and mast cell mediated cytotoxic killing. N denotes the number of experiments were repeated in each series. NS means not significant.

Table 2B. In vitro human mast cell-mediated cytotoxicity % (percentage based killing) measured by FCM-MCMCA against human LAK-sensitive tumor cells at different ratios and time periods (2h- 24h) are shown.

Percentage Based Human Mast Cell- Mediated Cytotoxicity (%, PC)						
Cell characteristics		**%, PC on Coincubation Times**				
LAK-sensitive	**Death Type**	**2h**	**12h**	**18h**	**24h**	**48h**
Effector (**Mast**) control/alone	Spontaneous	2±1	4±1	3±1	5±1	7±1
Target (**K562**) control	Spontaneous	3±1	4±1	5±1	6±1	5±1
Coincubation (2:1)	Cytotoxic	0±1	6±1	6±1	14±1	16±2
	N=	(3)	(9)	(9)	(9)	(9)
	P=	NS	NS	NS	NS	NS
Effector control	Spontaneous	4±1	3 ± 2	3 ± 2	4 ± 2	4 ± 2
Target **(Daudi**) control	Spontaneous	4±1	3 ± 1	4 ± 1	5 ± 2	8 ± 2
Coincubation (2:1)	Cytotoxic	30±1	57± 2	59± 2	63 ± 3	65 ± 3
	N=	(3)	(9)	(9)	(9)	(9)
	P=	**0.030**	**0.001**	**0.001**	**0.001**	**0.001**
Effector control	Spontaneous	5±1	2 ± 1	2 ± 1	4 ± 1	4 ± 1
Target (**Raji**) control	Spontaneous	7±1	5 ± 1	7 ± 1	9 ± 3	10 ± 3
Coincubation (2:1)	Cytotoxic	19±1	26 ± 1	46 ± 1	67± 3	75± 3
	N=	(3)	(9)	(9)	(9)	(9)
	P=	**0.044**	**0.016**	**0.006**	**0.001**	**0.001**
LAK-resistant	**Death Type**	**2h**	**12h**	**18h**	**24h**	**48h**
Effector (**Mast**) control/alone	Spontaneous	5±1	3±1	4±1	6±1	8±1
Target (**DAMI**) control/alone	Spontaneous	2±1	5±1	5±1	7±1	7±1
Coincubation (2:1)	Cytotoxic	6±1	15±2	17±2	20±2	22±2
	N=	(3)	(6)	(6)	(6)	(6)
	P=	NS	NS	NS	NS	NS
Effector control	Spontaneous	3±1	5±1	4±1	6±1	6±1
Target (**Meg-01**) control	Spontaneous	1±0	6±1	5±1	8±1	8±1
Coincubation (2:1)	Cytotoxic	4±1	14±2	18±2	22±4	25±4
	N=	(3)	(5)	(5)	(5)	(5)
	P=	NS	NS	NS	NS	**0.039**
Effector control	Spontaneous	3±1	6±1	3±1	5±1	5±1
Target (**HL-60**) control	Spontaneous	5±1	7±1	8±1	9±2	11±2
Coincubation (2:1)	Cytotoxic	5±1	13±1	16±1	28±3	36±3
	N=	(3)	(6)	(6)	(6)	(6)
	P=	NS	NS	NS	**0.034**	**0.019**
Effector control	Spontaneous	3±1	2±1	4±1	6±1	6±1
Target (**Patients**`) control	Spontaneous	2±1	6±1	8±1	10±1	13±1
Coincubation (2:1)	Cytotoxic	5±1	15±1	18±1	20±2	21±2
	N=	(6)	(6)	(6)	(6)	(6)
	P=	NS	NS	NS	NS	NS

The death/killings (percentage based cytotoxicity) in this table reflect the death at a 2:1 ratio in this study. Spontaneous kill reflects the death in target or effector alone tubes. Cytotoxic death shows the target cell death by only mast cell-mediated cytotoxicity in the coincubation sample. The percentage based cytotoxicity was calculated should be given formula. All values are given as mean±SEM. P values reflect the significance of differences between spontaneous and mast cell mediated cytotoxic killing. N denotes the number of experiments were repeated in each series. NS means not significant.

Table 2C. In vitro human mast cell -mediated cytotoxicity (%, PC) against different human tumor cells measured by both cytotoxicity assays is shown at various periods.

Cell Types	Percentage Based Human Mast Cell- Mediated Cytotoxicity (%)					
	12h			24h		
	Early Apoptotic	**Late Apoptotic**	**Total Killing**	**Early Apoptotic**	**Late Apoptotic**	**Total Killing**
Daudi	7%	50%	57 %	23%	40%	63 %
Raji	8 %	18%	26 %	27%	40%	67 %

Table 2D. The distribution of mean killing (%, PC) measured by FCM-MCMCA in human LAK-sensitive tumor cells at 2:1 ratio on different coincubation times in a representative sample.

In the literature, MCC did not seem to be very effective against some types of LAK-sensitive tumor cells, such as K562 and YAC-1 (Henderson, 1981; Ghiara, 1985; Richards 1988).Consistently, there was not significant killing (18% killing at most) in NK-/LAK-sensitive K562 cells even with 5:1 ratio at 48h (Table 2A,C). These findings supported well-known resistance to MCC by some LAK-sensitive cells. However, human MCC for first time was found to be very effective against different type of LAK-sensitive cells, such as Daudi and Raji, in this study. As shown in these studies, most of the total killing of both human target cells was necrotic kill and it increased over time. Interestingly, Raji killing, especially necrotic, apparently maximized at 24h, even though Daudi cell killing stayed almost stable and peaked at 12h. Both LAK-sensitive Daudi and Raji cell death were statistically significant between 2h-48h, compared to spontaneous killing. In vitro human MCC against LAK-sensitive cells at different ratios/times was shown in table 2B-C.

Moreover, in this study the FCM-MCMCA method allowed us to separate cytotoxic killing into different stages, early and late apoptotic (necrotic) kill. And distribution of apoptotic type killing according to the cell lines at 12h and 24h was shown at 2:1 ratio (Table 2D). Early apoptotic cell death up to 46% was also detected in the representative samples (Fig.1H2,I2), indicating the role of pro-apoptotic components of MC granules in human MCC.

Although at 2h/12h/18h there was some killing, statistically significant killing in LAK-resistant cells such as Meg-01 and HL-60 cells was demonstrated with both 2:1 and 5:1 ratios at 48h (Table 2A,C). In HL-60 cells, there was a statistically significant degree of kill at 18h (ratio 5:1) and 24h (ratio 2:1) as well. Moreover, the AML patient samples seemed to be very resistant to MCC. In patient cells, there was a fair amount of killing (≤25 %) but was not statistically significant (Table 2A,C). Insignificant killing in patient samples and LAK-resistant cells, probably due to small number of sample, but it was noticeably important. This is the first study demonstrating human MCC against some human LAK-resistant cells too.

After 2h/12h coincubations, neither at 2:1 nor at 5:1 ratios was there significant killing in any experiments except for Daudi and Raji cells. In LAK-sensitive Daudi/Raji cells, statistically significant death at different rates was demonstrated and remained significant from 2h-48h coincubation (Table 2B-C). Concurrently, short term MCC (≤18h) was observed in patient samples as well as DAMI/HL-60/Meg-01 cells in this study, although MCC in the long term is well-known. We even observed some degree of cytotoxicity in very short-term incubation (2h) in DAMI/Meg-01 cells, although there was not statistically significant killing. The killing detected between 2h-48h in all LAK-resistant cells including patients'

was close to statistical significance, partly due to an inadequate number of experiments (Table 2A,C). In overall; Daudi/Raji/Meg-01 and HL-60 cell deaths were found to be statistically significant at different periods in long term, compared to spontaneous (control) killing (Table 2A-C). Thus, these findings reveal human MC`s cytotoxic capacity against human LAK-resistant/-sensitive cells.

Briefly, interestingly, MCs did not seem to be very effective against some type of human NK-/LAK-sensitive cells such as K562; but LAK-resistant cells including Meg-01/HL-60/DAMI/patients' were killed somewhat effectively by MCC.

The ability to study MCC in longer coincubation times (≤48h), in addition to shorter coincubation times (2h-18h), is another advantage of our established FCM methods in this study. This appears to be a drawback for CRA as well as other methods utilizing dyes such as fluorochromes (PKH-26) due to increased risk in release of ^{51}Cr or dye leakage, which results in staining of other populations. Nevertheless, our applications are potentially important for studying certain apoptotic pathways that take longer to become operational, such as membranous TNF-α-induced apoptosis, which is believed to be one of most important components of MCC. Membranous TNF-α has been shown to kill WEHI-164/L929/Raji cells in 24h assays (Henderson, 1981; Ghiara, 1985; Richards 1988; Heikkilä, 2008; Özdemir, 2003, 2006).

In this study, our results were found to be reproducible and reliable when experiments repeated (mean coefficients of variation (CV) of 1.1%). Incremental increase in MCC detected at different ratios/times shows reproducibility and reliability of our methods (Table 2A-C). When our flow cytometric data compared with CRA results, there was a significant correlation ($n=104$, $r=0.82$, $p<0.001$), similar to our earlier studies (Özdemir, 2003, 2007, 2011).

In addition, Wright-Giemsa slides showed conjugate formations between MC and some tumor cells (MC-target cell doublets), indicating possible initial steps of human MCC, perhaps via cell-to-cell contact thru their membranous components such as membranous TNF-α/Fas Ligand (Fig.1A1-4).

4. Discussion

This study first of all demonstrated human MCC against human tumor targets by two different approaches that were very comparable to CRA. These are established and reliable methods elucidating MCC by a two- and three-color FCM assays using DIOC18 and mAb target-cell marking, respectively. Our research experiences with these methods have been recently published (Özdemir, 2003, 2007, 2011).

As mentioned above, murine MCC against lysis-sensitive murine tumor cell lines (WEHI–164/L929) in long term was previously well demonstrated (Henderson, 1981; Ghiara, 1985; Richards 1988). Nevertheless, to the best of our knowledge, this is the first study using human BM-derived effector MCs against human tumor cells to show and verify short- and long-term human MCC in vitro. Even significant kill in different LAK-sensitive/-resistant cells was also demonstrated for the first time. MCC was shown to be effective against different types of NK-/LAK-sensitive Daudi/Raji cells, but not to K562 cells or LAK-resistant cells. These findings are consistent with earlier murine studies demonstrating murine MCC against lysis-sensitive cells. Like murine cells, Daudi/Raji are also known to be somewhat lysis (TNF-α)-sensitive, and this explains statistically significant and mostly

necrotic killing in Daudi/Raji, which probably reflects pro-necrolytic characteristics of MCC.

Early cell death of LAK-sensitive/-resistant cells in our experiments detected from 2h indicated human MCC occurring in short-term coincubation. These results strongly suggest a possible contribution of a fast-acting secretory pathway via the exocytosis of pro-apoptotic granules of MCs to the human MCC. Accordingly, these findings verify the recent literature suggestive of MC-induced apoptosis in smooth muscle cell, cardiomyocytes and endothelial cells (Leskinen, 2003; Heikkilä, 2008).

The killing at various coincubation times in all LAK-resistant cells including patient cells was close to statistical significance partly due to an inadequate number of experiments. However, it was still noticeably important since these cells are known to be resistant to even LAK cells, and they are not essentially TNF-α sensitive cell. Thus our results also indicate a role for MC as a contributory effector cell in cellular immune surveillance of human innate immunity, resembling recently reported studies (Marshall, 2004; Özdemir, 2006).

Contrary to the findings of this in vitro study, MC availability in tissues and tumor stroma has still been controversial. The important point is here: increased tissue MCD could be primary and/or secondary since MCD is also found to be increased physiologically around healing tissue (Özdemir, 2006). MCs might be just as a reflection of generalized inflammatory reaction as well. For instance; the infiltration of MCs was thought to be a reflection of the host inflammatory response and is favorable prognostic factor in diffuse large B-cell lymphoma (Hedström, 2007). Similarly, MCs were believed to represent reactive cell types involve in the pathophysiology of the host reaction in lymphoma (Sharma, 1992). MCs also accumulate at sites of tumor growth in response to numerous chemoattractants or mediators. For example, CCL5/CXCR3 chemokines in lymphoma and tumor-derived stem cell factor and CD30 expressions lead to tumor growth and MCD in tumor tissues (Fischer, 2003). While evaluating the tumor-MC relationship, not only the MCD, but several other factors, such as the relationship of MCs with other stromal (fibroblasts, endothelial) and inflammatory cells should also be considered (Aldinucci, 2010). Hence it is important to find an answer to the following question: can the increased MCD be a result of tumor progression or a cause of tumor progression and a poor prognosis? Then the next question becomes, is the MC really an active player or an innocent passerby in a tumor stroma?

Increased MCD in the tumor stroma was assumed to be a stimulator of tumor progression through angiogenesis. As with other tumors; there are also conflicting results about the role of MCD and its relation to microvessel density (MVD) in lymphoma/leukemia (Özdemir, 2006). In addition, lymphoma progression is recently considered to be potentiated by at least two distinct angiogenic mechanisms. Autocrine stimulation of tumor cells via expression of vascular endothelial growth factor (VEGF) and VEGF receptors by lymphoma cells, as well as paracrine influences of proangiogenic tumor stroma on both local neovascularization and recruitment of circulating BM-derived progenitors (Ruan, 2009). Similarly, isolated AML blasts were shown to overexpress the VEGF/VEGFR–2 pathway, which can promote the growth of leukemic blasts in an autocrine and paracrine manner (Mesters, 2001). Yet, in some literature the following are hematological tumors in which MCD correlates with MVD: B-cell non-Hodgkin lymphoma, HL, follicular/angioimmunoblastic T-cell lymphoma, and B-cell chronic lymphocytic leukemia (Ribatti, 1998; Fukushima, 2001; Ribatti, 2009; Tripodo, 2010; Taskinen, 2010). And human tumors in which MCD is supposed to correlate with a poor prognosis include HL, diffuse large B-cell lymphoma, follicular and angioimmunoblastic T-cell lymphoma (Molin, 2002; Gratzinger, 2007; Ribatti, 2009;

Taskinen, 2010;). Amazingly, in some studies, human tumors in which MCD is supposed to correlate with a good prognosis include HL, diffuse large B-cell lymphoma, and follicular lymphoma (Koster, 2005; Hedström, 2007; Rygoł, 2007;Ribatti, 2009;). Although it is hard to explain these conflicting results above and in the literature, they may be associated with different methodologies used in studies such as timing of biopsy (e.g. doing in early instead of late stage of tumor), the tumor type, as well as environmental factors surrounding that tumor (Özdemir, 2006). Only observing increased MCD in various tumors with good or bad prognosis on pathological specimens seems to be far behind to explain the real role of MCs. The net effect of MCs on tumor growth, therefore, is likely to be the result of multiple interactions between MC, tumor, and associated inflammatory cells with their signaling pathways, and adjacent stromal cells such as vascular endothelium and fibroblasts (Ribatti, 1998; Özdemir, 2006; Ribatti, 2009; Aldinucci, 2010).

Consequently, antiangiogenic strategies have recently become an important therapeutic modality for solid tumors. And MCs have been thought of as a new target for the adjuvant treatment of tumors through the selective inhibition of angiogenesis, tissue remodeling and tumor promoting molecules. Preliminary studies with cromolyn in mice mammary adenocarinoma and pancreatic cancer therapy was ineffective. Although some therapeutics like alemtuzumab were also given as an example of antiangiogenic treatment success, it is known as an anti-myeloid cell antiangiogenic agent used for the treatment of ovarian cancer, not an anti-MC agent (Ruan, 2009). Beside MCs and myeloid cells, Tie2-expressing monocytes and vascular leukocytes have recently been shown as new targets in the regulation of tumor associated angiogenesis (De Palma, 2007). In the age of targeted therapy, studies of the targeting MCs` role in cancer might have direct clinical consequences and should be further elucidated via the use of histopathological and complex biological models.

Since the aim of this study was basically to show MCC using human effector/target cells, the mechanisms and specificity of human MCC were not evaluated in this study. Our studies still continue studying MC-tumor cell relations and the characteristics of MCC on human pathological biopsy specimens. We hypothesize that human MCC may happen thru non-secretory pathway (cell-to-cell contact) of membranous TNF-α/Fas L, and secretory pathway via released mediators like soluble TNF-α, granzymes and chymase. Simply, MCC happens via pro-apoptotic/-necrolytic granules of MCs in the short term, as well as through its membranous components (non-secretory pathway of MCC) in the long term as shown in this study. Consistently, observing effector-target doublets on Wright-Giemsa slides of this study might suggest the importance of cell-to-cell contact in this process. The mechanisms with characteristics of human MCC against human tumor cells, as well as the role of MC mediators, are discussed by us in detail somewhere (Özdemir, 2006).

5. Conclusion

Our findings in this study verify possible anti-tumor role of MC as a contributory effector cell to NK and other cytotoxic cells in immunosurveillance within human innate immunity. As current literature is still very confusing regarding MC`s role in and around the tumor tissue, this in vitro study may help enlighten its interactions with tumor cells and trigger new explanatory in vitro/in vivo studies like, for example, delineating the killing mechanisms of MCC.

These conflicting reports might indicate that there seem to be other factors determining the relationship between MCD and tumor progression. The conflicting results may also depend on wide variations in timing, tumor types/stages, methodologies, and the chemotherapy application as well. Importantly, some studies are not able to reflect the direct effects of MCs on tumor biology because many patients receive adjuvant chemotherapy in the time of sampling. So, some of the effects can be accounted for by the therapeutic response to chemotherapy. Nonetheless, by only observing increased MCD in and around a tumor, making a correlation between good or bad prognosis and specimens seems inadequate to explain their real role.

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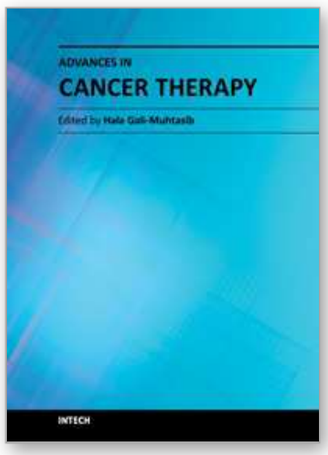

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