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Gene Expression in Human Acute Cutaneous and Hepatic Graft-*Versus*-Host Disease after Allogeneic Bone Marrow Transplantation

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Abstract. *Hematopoietic stem cell (HSC) allograft can be performed with cells of peripheral or medullar origin. Currently, it is the best therapy for certain malignant diseases. The curative power of allografts is based on conditioning and on the graft versus leukemia (GVL) effect. This effect is always associated with a toxic reaction called graft-versus-host disease (GVHD). Numerous studies have been carried out on mouse models, but the pathophysiology of GVHD remains unknown. To evaluate the variation in gene expression during GVHD, a prospective study was performed on two patients with GVHD, using the donors as controls. Blood lymphocytes were isolated by Ficoll gradient. The gene expression levels in total RNA were determined using the Taqman method. The gene expressions of cytokines* ***(TNFα, INFγ, IL4, IL10)****, major histocompatibility complex class II* ***(MHC II)*** *and class III* ***(BAT2),*** *an adhesion molecule* ***(VCAM)*** *and granzyme M were studied.* ***INFγ, TNFα, BAT2*** *and* ***IL4*** *were up-regulated whereas* ***IL10*** *and* ***VCAM1*** *were down-regulated.*

Hematopoietic stem cell (HSC) allograft of peripheral or medullary origin is a recognized treatment for some malignant hemopathies, and is under investigation for solid tumors and auto-immune diseases. The curative power of HSC allografts depends on both the conditioning, generally chemotherapy, with or without associated radiotherapy, and on the antitumor effect of the graft: GVL (graft *versus* leukemia) or GVT (graft *versus* tumor). This therapeutic effect is unfortunately often associated with a toxic reaction termed graft-*versus*-host disease (GVHD) which constitutes a major complication of allogenic treatment. The usual clinical signs are cutaneo-mucous, digestive and/or hepatic. Corticoids are the major drugs in the treatment of this complication. Several other immunosuppressors have been used with variable results. In the majority of serious cases, GVHD therapy remains a challenge and may become serious if treated too late. A prospective study was performed on two patients and their donors to evaluate the relative quantity of messenger RNA of different genes at the moment of GVHD.

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Patients and Methods

Patients and donors. Two donor/receiver couples were the subject of the study. When GVHD occurred, the hematological reconstitution was allogeneic, with the donor lymphocytes being responsible for the GVHD. The patients fulfilled the following criteria: intra-familial allograft, hematological malignancy in complete remission, homogeneous conditioning, homogeneous prevention of GVHD, age at least 18 years and IBMTR or Glucksberg grade < II GVHD.

Blood sampling. Blood samples were taken from the recipients during the end of aplasia and at the time of the first GVHD event. Neither patient had received immunosuppressive treatment, other than cyclosporin prescribed for disease prevention. Lymphocytes were collected from one patient before any clinical evidence of GVHD.

To avoid excessive discomfort, donor lymphocytes were collected at the same time as the peripheral blood stem cells. To ensure the absence of inflammatory activation at the time of harvest, inflammatory parameters were evaluated. To isolate lymphocytes, 20 ml of blood were collected in heparin tubes and the lymphocytes were isolated on Ficoll (d=1.077) by standard procedures.

Total RNA extraction and quality control. The lymphocytes were resuspended in 3 ml of RNA-PLUS2™ (Q-BIOgene, Illkirich-Graffenstaden, France). The homogenate was collected, 1 ml of chloroform added, followed by agitation for 15 seconds. After 30 minutes centrifugation at 12,000 xg, the aqueous phase containing the RNA was collected. One volume of isopropanol was added to precipitate the RNA overnight at 4˚C. Following 30-minute centrifugation at 12,000 xg, the RNA pellet was washed with 3 ml of 75% ethanol, then dried at ambient temperature before resuspension in 50 µl DEPC water. Once solubilized, the RNA was analyzed by spectrophotometry and stored in liquid nitrogen.

The RNA quality was assessed at 260 nm and 280 nm by absorption on a Bioanalyzer 2100 (Agilent®, Wilmington, USA). For each sample, 1 µl of RNA was diluted to 300 µg/ml then deposited on an analysis chip. Electrophoretic migration separates the RNA by size, the migration profile being detected by a laser. The RNA was considered to be of good quality when the ratio of *28S* to 18S *rRNA* was close to 2.

Reverse transcription of RNA. Five µg of RNA was retro-transcribed into cDNA using the First-Strand DNA Synthesis Kit (Amersham Biosciences, Uppsala, Sweden) and random hexanucleotide primers, according to the manufacturer's instructions.

Real-time Taqman RT-PCR. The mRNA for eight genes was quantified*: BAT2, MHC II, TNF, INFγ, IL10, IL4, GZMM* and *VCAM1* by quantitative real-time Taqman RT-PCR. This quantification was carried out on total lymphocytes in order to observe variations of the expression of these genes in the presence of GVHD. Each reaction was performed in triplicate. The mRNA levels were quantified using an ABI Prism 7700 Sequence Detection System (Applied Biosystems®, ZA, Courtaboeuf, France) and Assay-on-Demand® (Applied Biosystems®) except for *18S,* the probes and primers for which were designed with the help of Primer Express (Applied Biosystems®). The sequences are presented in Table I. Calculation of the relative quantities of mRNA was carried out using the mathematical formula: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ Patient – ΔCt Donor and ΔCt = Ct of studied gene – Ct of the *18S* gene. Thus, it was possible to obtain standardized results and to determine the relative quantity of mRNA of the patient compared to the donor. Statistical analysis was carried out by Student's *t*-test and variations were considered significant when $p<0.05$.

Table I. *References of the Assay-on-Demand® Kits used (Applied Biosystems) for the quantification of mRNA of TNFα, INFγ, MHC II, BAT2, IL4, IL10, GZMM and VCAM1 by Taqman® real-time quantitative RT-PCR.*

Studied genes	Abbreviations	References Assay-on-Demand®
Tumor necrosis factor α	*TNFα*	Hs.00174128
Interferon γ	*INFγ*	Hs.00989291
MHC class II transactivator	*MHC II*	Hs.00172106
HLA-B associated transcript 2	*BAT 2*	Hs.00190347
Interleukin 4	*IL4*	Hs.00174122
Interleukin 10	*IL10*	Hs.00174086
Granzyme M	*GZMM*	Hs.00193417
Vascular cell adhesion molecule 1	*VCAM 1*	Hs.00365486

Results

*Quantification of **INFγ, TNFα, BAT2, MHC II** transactivator, **VCAM1, GZMM, IL4** and **IL10** mRNA for the patient with cutaneous expression (Figure 1).* No difference in *INFγ* expression was found at the end of aplasia compared to the donor. However, a significant increase ($p<0.05$) in *INFγ* mRNA expression was detected once this patient presented cutaneous signs of GVHD. Further, no change in *TNFα* expression at the end of aplasia was observed compared to the donor, although a statistically significant increase ($p<0.05$) in *TNFα* mRNA expression was found once the patient presented with signs of cutaneous GVHD. The increase in the *BAT2* expression was significant at the end of aplasia without any clinical signs and during GVHD. A significant increase in *MHC II transactivator* mRNA was observed at the end of aplasia ($p<0.05$). Additionally, a significant up-regulation of *VCAM1* mRNA expression was observed compared to the donor ($p<0.01$). No variation in the form of the *GZMM* gene was detected. The increase of *IL4* gene expression was important, variations appearing at the end of aplasia and onset of clinical expression, although the increase was lower during clinical expression. Finally, inhibition of *IL10* expression was observed ($p<0.0005$).

*Quantification of **INFγ, TNFα, BAT2, MHC II** transactivator, **VCAM1, GZMM, IL4** and **IL10** mRNA for the patient with hepatic expression (Figure 2).* The patient's *INFγ* expression was slightly increased at the end of aplasia, but significantly increased at GVHD which corresponded to a J-60 post allograft ($p<0.05$). Biological disturbances had begun from the end of aplasia. At this time, a significant increase in *TNFα* gene expression had been noted ($p<0.01$) and also at GVHD ($p<0.01$). Furthermore, a significant increase ($p<0.01$) in *BAT2* expression was noted at GVHD. Compared to the donor, the *GZMM* gene was significantly down-regulated at the end of aplasia. For *IL10*, a significant increase ($p<0.001$) was found only at GVHD.

Discussion

GVHD is an immune reaction started by donor lymphocyte recognition of alloantigens which are presented by antigen presentation cells (APC) to naive donor T cells. The T

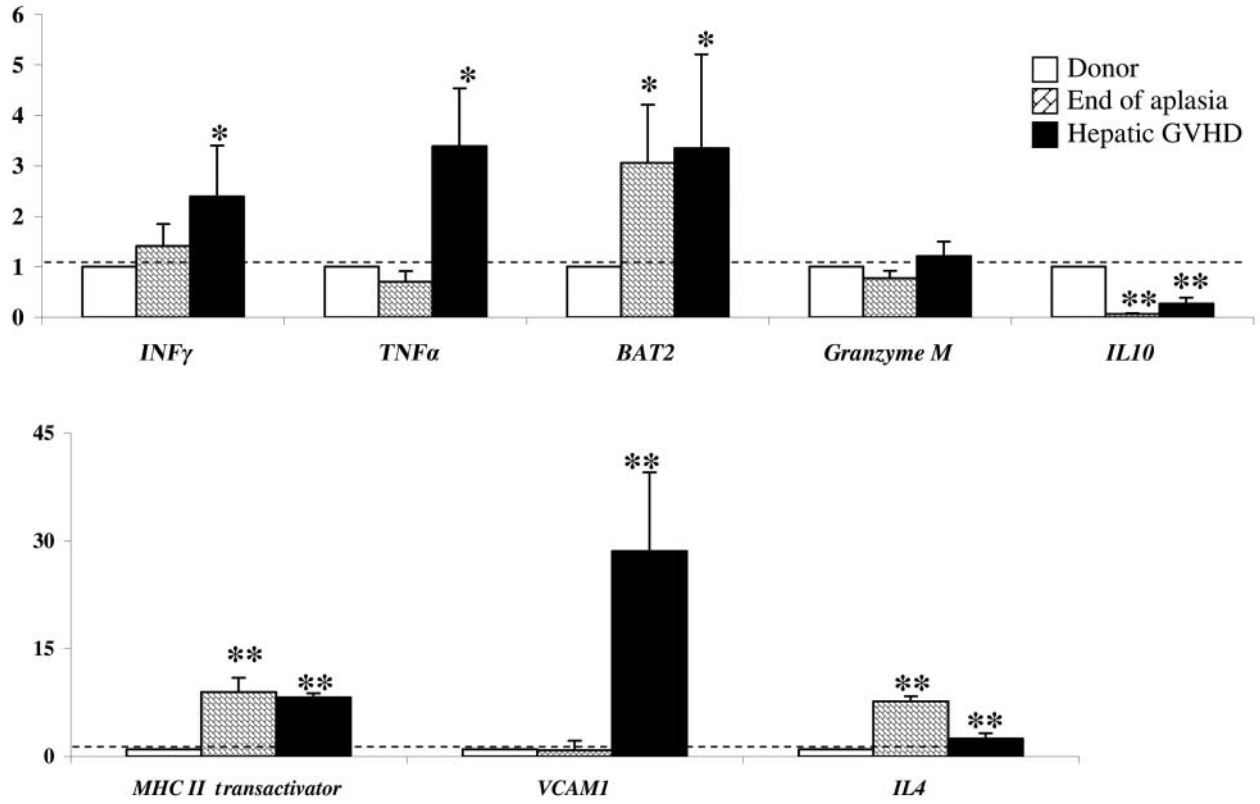

Figure 1. *Determination of INFγ, TNFα, BAT2, granzyme M, IL-10, MHC II transactivator, VCAM1 and IL4 relative mRNA expressions using the Taqman® real-time RT-PCR method in total lymphocytes from a cutaneous GVHD case.*

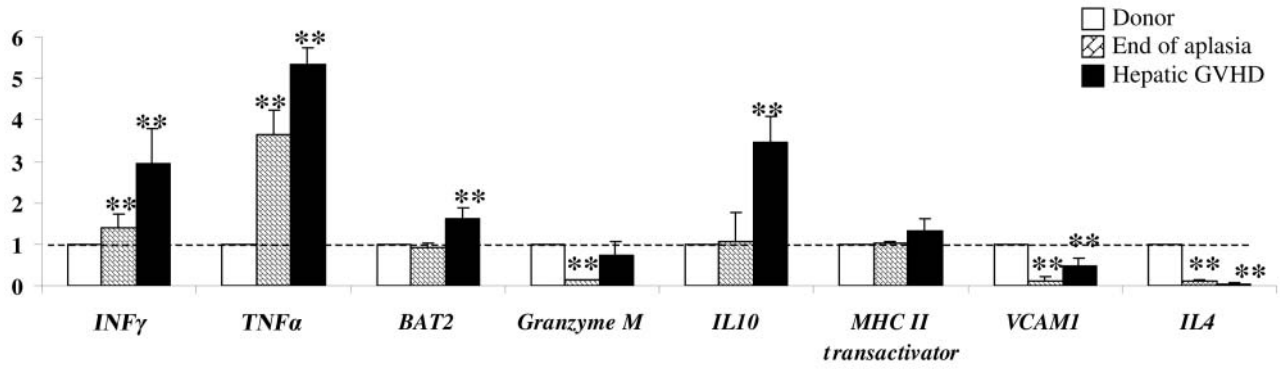

Figure 2. *Determination of INFγ, TNFα, BAT2, granzyme M, IL10, MHC II transactivator, VCAM1 and IL4 relative mRNA expressions using the Taqman® real-time RT-PCR method in total lymphocytes from a hepatic GVHD case.*

lymphocytes in turn produce Th1 or Th2 cytokines, responsible for starting the cytokine cascade. Th1 lymphocytes secrete ***IL2*** and ***INFγ*,** essential mediators of GVHD (1).

An increase in serum ***INFγ*** levels at the time of GVHD has already been shown (2). ***INFγ*** is responsible for inflammatory cytokine secretion by macrophages and for cutaneous and digestive attack (3). In this study, the expressions of certain cytokines in total lymphocytes from patients with cutaneous or hepatic GVHD were evaluated. Blood samples from the patients were taken at the first

clinical sign of GVHD, with their donors being used as controls. All lymphocytes taken from patients were of donor origin, as determined by the evaluation of chimerism (data not shown). Samples were taken before and after the appearance of clinical signs in the patient presenting with cutaneous GVHD and at the start of hepatic biological disturbances in the patient presenting with hepatic attack.

It has been established that GVHD is the major obstacle to hematopoietic stem cell transplantation (HSCT). Its physiopathology remains unknown and therapeutic tools are primarily based on immunosuppressive therapies that remain ineffective for grades III and IV. Most research has been done on murine models using flow cytometry and the first results of genomic analysis by microarrays were recently published. In one model, the mice received an HLA minor and major histocompatibility antigen, *INFγ*. Inducible genes such as *STAT1, IRF-1, IIGP, GTP1, IGTP, IFI202A, MHC II* and genes related to leukocyte trafficking were up-regulated (4). *INFγ* itself was not expressed. This expression preceded hepatic lymphocytic infiltration. Surgerman *et al.* (5) reported the same observation on cutaneous mouse samples.

In our study, *INFγ* seemed to be significantly increased in both patients. A previous study revealed an early increase of ***INFγ*** after hematopoietic stem cell transplant (HSCT), particularly in the spleen (6). However, splenectomized mice were not protected from hepatic GVHD (7). INFγ may be released elsewhere. Macrophages and monocytes produce TNFα, IL1 and INFγ (8, 9). TNFα is one of the first cytokine cascades in GVHD (10). In this study, *TNFα* up-regulation in two patients at the time of biological and clinical disturbances was significant. This increase was strongly GVHD-related, and was not found in the absence of clinical signs in the patient with cutaneous expression. Apart from its role as an inflammatory cytokine shared with INFγ, TNFα is also an inducer of apoptosis.

An aberrant expression of the molecules *LFA-1, ICAM-1* and *VCAM-1* in bile ducts at the moment of hepatic GVHD has also been reported (11). This event at D-7 precedes hepatic infiltration by effector cells observed from J-35 (4). *VCAM-1* (CD106) was expressed normally in the myeloid line and in the endothelial cells, allowing leukocyte migration towards an inflammatory site (12). This *VCAM-1* up-regulation was also observed at D-7 in cutaneous samples or keratinocytes, attesting to the role of *VCAM-1* in cutaneous GVHD (5). The gene expression kinetics and cutaneous histological modifications appeared to be correlated.

In our study, the *VCAM-1* expression of the cutaneous GVHD patient was highly up-regulated in comparison to that of the donor and also in comparison to periods before and after the appearance of clinical signs. However, this up-regulation was not found in the case of the hepatic GVHD patient.

Major histocompatibility complex (MHC) expression is restricted to monocytes and B lymphocytes, both recognized by T CD4 lymphocytes. They mainly present exogenous antigens, in particular alloantigens. However, T CD8 lymphocytes interact with class *I MHC* at the time of GVHD presentation, and aberrant class *II MHC* expression in bile ducts has been reported, constituting the target of the effector cells (13). T CD4 lymphocytes seem to be incriminated in chronic GVHD, especially when there is a class II mismatch (1). T CD8 lymphocytes are responsible for GVHD in the event of incompatibility in the minor system or class *I MHC* system. In our study, the *MHC II transactivator*, a main regulator of class *II MHC,* did not seem to interfere at the point of hepatic disturbance. It was, however, strongly expressed at the time of cutaneous GVHD.

T CD4 lymphocyte development and activation play important roles in infection, auto-immune and immuno-allergic diseases. Their repertory of cytokines is very distinct (14). Th1 cells primarily secrete ***INFγ*** and ***IL2,*** incriminated in auto-immune diseases. Th2 cells produce *IL4* and *IL5,* involved in allergic diseases (15). It was demonstrated that *IL4*-producing cells inhibited the development of acute GVHD (16) and that *IL4*-producing CD8(+) T cells may be an immunological marker of chronic GVHD (17). This form is closely related to T CD8+ reduction directed against the recipient. In the patient with cutaneous GVHD, *IL4* expression increased significantly as early as hematological recovery. This increase lessened with the second blood sample at the time of cutaneous GVHD expression. This patient currently suffers from a chronic cutaneous and mucous attack. No modification of the *IL4* expression level was found in the patient with hepatic GVHD.

It has been shown that Th1/Th2 polarization plays an important role in the development of acute GVHD (18). The factors involved in Th1 or Th2 polarization are primarily the type of signal activating dendritic cells (DCs) and the ratio of DCs to T cells. Th2 cells have a suppressive action. Natural killer 1T (TNK1) can inhibit GVHD by producing *IL4 (19)*. The capacity of Th2 cytokines to polarize T lymphocytes and to reduce the severity of GVHD was shown in many animal models (18, 20). Polarization was achieved by the administration of IL4 to donors (21).

The final step of acute GVHD is target organ destruction by induction of apoptosis. Several pathways are involved such as Fas in hepatic attack, TNF in digestive attack (22) and TNF, Fas and granzyme B in the cutaneous form.

The cytotoxic role of ***granzyme M*** (GZMM) has not yet been established (23), although, the role of granzymes A and B in apoptosis induced by cytotoxic lymphocytes is recognized. GZMM is found primarily on T CD3+ and

T CD56+ cells, suggesting a role for GZMM in the inflammatory reaction. In our study, *GZMM* was significantly up-regulated in hepatic GVHD ($p<0.01$), while it remained stable in cutaneous GVHD.

BAT2 is localized to 6p21.3 within the *MHC III,* near *TNFα* and *TNFβ*, but its function is unknown. *BAT2* interacts with the protein C1q binding complement. It is currently incriminated in inflammatory pathologies such as autoimmune diabetes and is regarded to be one of the candidate genes for rheumatoid polyarthritis (24). A significant increase in *BAT2* was observed in both our patients ($p<0.01$). In the patient with cutaneous attack, the increase was significant before and continued through the appearance of clinical signs ($p<0.01$).

Conclusion

The results obtained by Taqman® real-time RT-PCR demonstrated that *INFγ, TNFα, BAT2* and *IL4* were up-regulated, whereas *IL10* and *VCAM1* were down-regulated. This seemed to reproduce the observations in murine models, in spite of the complexity of our analysis. Patients have medical antecedents other than their hematological malignances, often being in receipt of chronic medications at the moment of blood sample analysis. The *IL10* and *BAT2* variations were unexpected and require further exploration. Our continuing investigations will involve the use of pangenomic transcriptome DNA chips in a large cohort of patients. In order to eliminate transplantation-related modifications of expression, it will be of interest to compare these results with those of donor/recipient couples without GVHD.

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