ORIGINAL ARTICLE

The detection of apoptosis in a human in vitro skin explant assay for graft versus host reactions

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Aims: Keratinocyte apoptosis is a major pathogenic mechanism in dermal complications, such as graft versus host disease (GVHD), after allogeneic bone marrow transplantation. However, the mechanisms by which recipient target cells undergo apoptosis in GVHD are still unclear, but may result from DNA damage caused by chemotherapeutic agents and/or by direct cytokine action. The basis of this investigation was to correlate keratinocyte apoptosis with (1) the severity of graft versus host reactions (GVHR) in vitro and (2) the clinical grade (0–III) of GVHD.

Methods: Skin sections generated from an in vitro skin explant model for detecting experimental or clinically relevant GVHR were investigated for the detection of apoptotic nuclei using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) technique. This investigation also aimed to establish whether the TUNEL assay could be used as an additional, predictive method for the severity of GVHD before transplantation in potential patient/donor pairs given standard GVHD prophylaxis (cyclosporin A and methotrexate).

Results: By comparing mean values of apoptosis for each GVHR grade in a cohort of 83 retrospective skin sections it was shown that as the severity of GVHR increased there was a parallel increase in the percentage of apoptotic cells ($p < 0.0001$). However, the correlation between clinical GVHD grade II–III and overall keratinocyte apoptosis ($> 2.6\%$) did not reach this degree of significance (χ^2 : 4.2; degrees of freedom, 1; $p = 0.04$; Fisher's exact test: $p = 0.06$).

Conclusions: The detection of apoptosis correlated with degree of GVHR using an in vitro assay and a higher degree of apoptosis tended to correlate with more severe GVHD. Further studies in a larger cohort of patients, using other methods to detect apoptosis in conjunction with the TUNEL assay, may give additional insight into the complex immunopathophysiology of GVHD.

Keratinocyte apoptosis (programmed cell death) is regarded as a major pathological mechanism of cutaneous complications, such as graft versus host disease (GVHD), following allogeneic stem cell transplantation. However, a direct causal relation still lacks experimental evidence.

The stimuli and mechanisms by which recipient target cells undergo apoptosis in GVHD are still largely unknown, but may be elicited by a variety of means, including DNA damage caused by chemotherapeutic agents, such as cyclophosphamide,¹ and direct cytokine action.² However, apoptosis in GVHD almost certainly involves receptor ligand interactions, including the tumour necrosis factor α (TNF- α) and its receptor p55. In particular, the transmembrane bound form of TNF- α is crucially involved in target cell apoptosis as a result of conditioning related toxicity³ and alloreactive primed monocytes.⁴ TNF- α interactions and monocyte involvement can also be initiated via activated donor T cells as a result of major histocompatibility complex (MHC) incompatibility.⁵ The infiltration of donor lymphocytes into target tissue demonstrates that cytotoxic T cells play a role in the initiation of apoptosis, either directly or via specific soluble mediators.⁶ Granzyme B, a product of cytotoxic T cell granules, also invades the target cell and initiates apoptosis via caspase 10.⁷ In addition to TNF- α , other soluble and membrane bound mediators, such as Fas ligand (FasL), can confer apoptotic cell death.⁸ Recent data also strongly suggest a target organ specificity of GVHD related tissue damage,⁹ and that the main target cells for GVHD in the skin are keratinocytes. The basis of our investigation was to correlate keratinocyte apoptosis with the severity of graft versus host reactions (GVHR) in skin sections generated from an in vitro skin explant model used as an experimental model to study GVHR, and to predict GVHD

in allogeneic stem cell transplants¹⁰ (for a recent review see Sviland and Dickinson¹¹).

"T cells play a role in the initiation of apoptosis, either directly or via specific soluble mediators"

We compared the histopathological evaluation of GVHR in patient skin biopsies with the demonstration of apoptotic nuclei using an immunohistochemical terminal deoxynucleotidyl transferase (TdT) dUTP nick end labelling (TUNEL) technique.¹² This investigation aimed to establish whether the TUNEL assay could provide a valuable, additional predictive method for the severity of GVHD before transplantation in potential patient/donor pairs given standard GVHD prophylaxis consisting of cyclosporin A and methotrexate.

MATERIAL AND METHODS

Skin explant assay for the detection of graft versus host reactions in vitro

The evaluation of GVHR and the detection of keratinocyte apoptosis were carried out on 83 retrospective skin sections, from 10 skin explant assays, including controls, together with a further 18 skin explant assays from patient and donor

Abbreviations: FasL, Fas ligand; GVHD, graft versus host disease; GVHR, graft versus host reactions; HLA, human major histocompatibility complex; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; SE, standard error; TdT, terminal deoxynucleotidyl transferase; TNF- α , tumour necrosis factor α ; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling

Figure 1 Diagrammatic representation of the human skin explant assay. GVHR, graft versus host reaction; HLA, human major histocompatibility complex; MLC, mixed lymphocyte culture.

Table 1 Histopathological representation of skin graft versus host reactions grades 0–IV	
Grade	Histopathology
0	Normal skin
I	Vacuolisation of epidermal basal cells
II	Diffuse vacuolisation of basal cells, with dyskeratotic bodies
III	Subepidermal cleft formation
IV	Complete epidermal separation

human MHC (HLA) matched sibling pairs where clinical outcome was known. In brief, the skin explant assay was carried out as described previously.^{10 11} The experiments consisted of responder cells from normal individuals or transplant donors being incubated for seven days with patient stimulator cells in individual mixed lymphocyte cultures (fig 1). Each individual experiment consisted of 1×10^7 responder peripheral blood mononuclear cells (PBMC) cultured with an equal number of irradiated patient PBMC in 10 ml of complete medium supplemented with 20% heat inactivated human AB serum in a 25 cm² culture flask (Nunclon, Roskilde, Denmark). After seven days of culture, 4 mm patient skin biopsies were dissected and explants were added to responder lymphocytes at a concentration of 1×10^6 cells/well in complete medium with 10% autologous serum to the skin biopsy.¹⁰ Control explants were incubated with medium alone or autologous lymphocytes alone.

After three days of co-culture, skin explants were fixed in 10% buffered formalin, paraffin wax embedded, and sectioned. For the purposes of the histopathological evaluation of GVHR, skin explants were stained with routine haematoxylin and eosin and evaluated according to the grading system described by Lerner *et al* (table 1).¹³ Grade 0–I changes were observed when skin explants were cultured with medium alone and were considered to be background. The correlation of GVHR and clinical GVHD was carried out using the criterion that grade II or above GVHR is a positive result and is predictive of grade II or above GVHD.

TUNEL for the detection of apoptosis

TUNEL is an immunohistochemical staining method that has been developed to enable the study of apoptosis in paraffin wax embedded tissue sections by light microscopy.¹⁴

Before sectioning, the glass slides were coated once with 2% triethoxysilyl propylamin (silane) (Merck, Poole, Dorset, UK) in acetone for five minutes, followed by washes in acetone and

Table 2 Comparison between the severity of graft versus host reaction (GVDR) in the skin explant assay and the degree of apoptosis seen for each GVDR grade 0–IV					
	GVDR grade				
	0	I	II	III	IV
No. of sections	1	38	15	28	1
Mean % apoptosis	0	1.1	2.7	4.5	12.3
Minimum	0	0	0	0	12.3
Maximum	0	4.8	13.3	15.8	12.3
Standard deviation	0	0.65	0.94	4.23	0
Standard error	0	0.22	0.85	0.79	0

Figure 2 Correlation between keratinocyte apoptosis and histopathological gradings of graft versus host disease in the skin explant model. As the severity of the graft versus host reaction (GVHR) increases, there is a corresponding increase in the degree of apoptosis observed (mean values are indicated by the bar). An unpaired *t* test comparing mean values of apoptosis between grade 0–I (regarded as background) and grade II–IV, at a 95% confidence interval, gave a *p* value of ≤ 0.0001 .

then distilled water (five minutes each). Slides were air dried at room temperature for 48 hours before sectioning. The paraffin wax embedded skin biopsies generated from the skin explant assay for the purposes of TUNEL were then sectioned in duplicate on the silane coated slides.

Excess paraffin wax was removed from the tissue by dewaxing the slides in xylene for five minutes. The tissue was then rehydrated by washing the slides for five minutes each in ethanol ($\times 2$), 95% alcohol, 70% alcohol, and distilled water.

Unless otherwise stated, all reactions were carried out at room temperature in a humid atmosphere. Cells were stripped of proteins and made permeable by incubation with 20 μ g/ml proteinase K solution (Sigma, Poole, Dorset, UK) for seven minutes, followed by an incubation with Triton-X-100 (BDH, Poole Dorset, UK) (0.1% Triton-X-100 in 0.1% citric acid, pH 3.0) for two minutes on ice. Slides were washed in distilled water for five minutes ($\times 3$). Sections were then pre-incubated with S1 buffer (330mM sodium acetate, 500mM NaCl, 0.3mM ZnCl₂, pH 4.5) for 10 minutes. The sections were then treated by immediately covering with 5 U/ml S1 nuclease (Roche Diagnostic, Lewes, East Sussex, UK) for 45 minutes at 37°C. Slides were then washed in distilled water for five minutes ($\times 3$), before being transferred to 1 $\times$ TdT buffer (200mM KCl, 25mM Tris, 5mM CoCl₂, 25 mg/ml bovine serum albumin, pH 6.8) for 30 minutes.

Sections were then incubated with TdT reaction mix (98 U/ml terminal transferase (Roche Diagnostic), 8 μ M biotinylated 16-dUTP (Roche Diagnostic) in 1 $\times$ TdT buffer) for 90 minutes at 37°C and covered with hydrophobic coverslips. The reaction

Figure 3 (A) Haematoxylin and eosin stained skin section from the skin explant assay showing a grade 0–I reaction. This is normal skin and considered as background. Mild vacuolisation of basal cells is seen, together with the occasional dyskeratotic body. (B) Haematoxylin and eosin stained skin section from the skin explant assay showing a grade III reaction. This is considered a positive result and a severe graft versus host reaction. (i) Diffuse vacuolisation of basal cells, with (ii) scattered dyskeratotic bodies, and (iii) subepidermal cleft formation. (C) Grade I terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) biopsy showing < 1% apoptotic cells (brown nuclei). Most of this skin section shows negative staining (blue counterstain). (D) Grade III TUNEL biopsy, showing 5.3% apoptotic cells (arrows).

was stopped by plunging the slides in 2× saline sodium citrate (330mM NaCl, 30mM sodium citrate) for two minutes, followed by washing in distilled water for five minutes (×2). Endogenous peroxidase was inactivated by covering the sections with 3% H₂O₂ (Sigma) in methanol for 20 minutes in the dark. The sections were then covered with a 2% aqueous solution of bovine serum albumin (BDH) for 10 minutes before incubation for 30 minutes with streptavidin–pox conjugate (Dako, Ely, Cambridgeshire, UK) (1/250 in 1× phosphate buffered saline). Slides were washed in distilled water (×3) before being immersed in DAB solution (0.625 mg/ml diaminobenzidine (Sigma), 0.03% H₂O₂, in 5mM Tris buffered saline) for five to 10 minutes. The sections were counterstained with Gill's no. 3 haematoxylin solution (Sigma) for 30 seconds, rinsed in tap water, and washed in distilled water for five minutes. Slides were finally mounted in glycerol (Dako) and assessed for the degree of apoptosis by light microscopic identification of brown stained nuclei in the epidermal layer.

Over 100 nuclei were counted for each slide and the percentage apoptosis in each section recorded and correlated with the histopathological grade of GVHR (0–IV).

TUNEL controls

Negative controls were provided for each section under evaluation by omitting the TdT enzyme from the TdT mix in the reaction. Positive controls were established by treating a skin section with DNase (2 µg/ml) (Roche Diagnostic) before incubation with 1× TdT buffer.

Statistical evaluation

The Student's unpaired *t* test, the Mann Whitney U test, χ^2 analysis, and Fisher's exact test were used for the correlation of GVHR with percentage apoptosis and the evaluation of results with patient data.

RESULTS

Correlation of the skin explant assay and percentage apoptosis

In this investigation 83 experimental, retrospective skin explants were evaluated for the severity of GVHR; 39 explants were found to give a histopathological grade of 0–I and were considered to be negative biopsies (background); 44 explants had a positive GVHR grade of II–IV. All 83 explants were stained for the detection of apoptosis using TUNEL and grouped according to GVHR grades I–IV. The mean degree of keratinocyte apoptosis was higher in explants demonstrating more severe GVHR (table 2; fig 2). The percentage apoptosis of GVHR grade I explants (mean, 1.1%; standard error (SE), 0.22%) was significantly lower than that observed for grades II–IV GVHR (mean, 4.2%; SE, 0.82%; *p* < 0.0001).

The high SE in percentage apoptosis for GVHR grades II (0.85%) and III (0.79%) highlight the variation in apoptosis noted in individual explants with GVHR grades II and III (table 2). In two of 29 cases, grade III explants showed no indication of apoptotic cells and three of 39 grade I explants showed apoptosis values greater than 4%.

Figure 3A and B shows typical grade 0 and grade III reactions, respectively, generated from the skin explant assay. Sections were stained with haematoxylin and eosin to allow morphological assessment. Figure 3C and D shows TUNEL staining on a grade I explant and a grade III explant, respectively. Brown stained nuclei are assessed as apoptotic cells, whereas blue stained cells are negative and are counterstained with haematoxylin.

Correlation between the skin explant assay, percentage apoptosis, and clinical outcome

A further 18 skin explant assays from patient biopsies were studied in which the clinical acute GVHD outcome was known. All of these HLA matched sibling patients had been given intensified GVHD prophylaxis consisting of cyclosporin

Table 3 A cohort of 18 patient/donor pairs comparing degree of graft versus host reaction (GVDR) from the skin explant assay, percentage keratinocyte apoptosis by TUNEL, and the clinical graft versus host disease (GVHD) outcome

Biopsy no.	Mean percentage apoptosis Medium control	Autologous lymphocyte control	Autologous MLR control	MLR	GVDR grade	Clinical GVHD grade
1	0.20	ND	3.00	6.15	III	0
2	0.00	0.20	0.20	0.20	III	0
3	1.60	ND	0.00	2.25	III	I
4	0.10	0.20	0.20	2.20	III	I
5	0.15	0.20	0.25	2.85	III	I
6	1.5	0.00	ND	5.00	II	II
7	0.00	ND	1.00	0.30	II	II
8	0.00	ND	3.49	6.55	III	II
9	0.06	ND	0.00	0.00	III	II
10	0.20	2.44	0.20	1.00	III	II
11	0.00	0.00	0.25	3.25	III	III
12	0.70	0.95	2.45	2.55	III	III
13	0.86	ND	0.00	2.90	III	III
14	0.20	0.20	ND	3.35	II	III
15	0.00	0.00	1.54	3.20	III	III
16	0.10	2.65	3.70	4.63	II	III
17	0.16	1.50	1.98	2.70	II	III
18	1.00	ND	ND	5.78	III	III
Mean values	0.38	0.76	1.22	3.04		

MLR, mixed lymphocyte reaction; ND, not determined; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

Table 4 Prognostic significance of graft versus host reaction measurement of keratinocyte apoptosis in a cohort of 18 patients receiving allogeneic grafts and intensified immunosuppressive prophylaxis

Graft versus host disease		
% Apoptosis	≤Grade II	Grade III
≤2.6	6	1
>2.6	4	7

Differences were analysed by χ^2 analysis and Fisher's exact test.

A and methotrexate. Of 13 patients where the skin explant was positive (grade II or above), all had clinical GVHD of grade II–III (table 3); the remaining five skin explant positive patients had clinical grade 0–I GVHD. A skin explant assay result of GVHR grade 0–I or a GVHR of grade II and above correlated with grades 0–I and II–IV of clinical GVHD in 72% of cases, although it did not correlate with actual grade II or III GVHD (Fisher's exact test: $p = 0.6$).

The mean (SE) percentage of keratinocyte apoptosis in the medium control was 0.38% (SE, 0.12%; table 3). The percentage of keratinocyte apoptosis seen in the autologous, lymphocyte control (mean, 0.76%; SE, 0.30%) was similar to the autologous mixed lymphocyte reaction (mean, 1.22%; SE, 0.35%). The GVHRs for these explants were all grade 0–I (data not shown). The mean (SE) apoptosis found in explants incubated in the presence of mixed lymphocyte responder cells was 3.04% (0.46%) and ranged from 0% to 6.55%, with a median of 2.60%. A percentage apoptosis above 2.6% was seen in 11 of 18 mixed lymphocyte reaction responder cell skin explant assays, all of which demonstrated grade II or above GVHR. Nine of these patients developed clinical GVHD grade II or above; seven developed more severe clinical grade III GVHD (table 4). Overall, keratinocyte apoptosis > 2.6% showed a general trend towards a positive correlation with GVHD grade III (χ^2 : 4.2; degrees of freedom, 1; $p = 0.04$; Fisher's exact test: $p = 0.06$).

DISCUSSION

Since results were first published by Berkman and colleagues¹⁵ using the skin explant assay as an in vitro model

and clinical predictive assay for GVHD,¹⁶ there have been several retrospective studies carried out relating GVHR in the skin explant assay to clinical outcome of acute GVHD in HLA matched allogeneic bone marrow transplants.^{10 11 17 18} In a report of a total of 34 patient/donor pairs, the overall correlation between the skin explant assay using responder cells and the development of clinical GVHD was 82%. The positive predictive value was 80% on GVHD grade II or above.¹⁷ In more recent studies, the skin explant assay, in both paediatric and adult cohorts, has successfully predicted GVHD in 80–90% of bone marrow transplant recipients.^{10 18} However, these positive results are dependent on the type of GVHD prophylaxis. The skin explant assay can be used to upregulate GVHD prophylaxis from cyclosporin alone to cyclosporin and methotrexate if the patient is predicted to develop GVHD and the skin explant assay is positive, with a grade II or above GVHR. Therefore, it follows that if the routine regimen for GVHD prophylaxis is cyclosporin plus methotrexate the skin explant assay is not such a strong predictive test.¹⁰ This effect was also noted in our present series of 18 HLA matched sibling donor transplants given cyclosporin plus methotrexate as GVHD prophylaxis. The skin explant results correlated with clinical outcome to the level of 72%. However, a skin explant GVHR grade II–III positive result did not correlate directly with GVHD grade in HLA matched sibling cohorts.¹⁹ Therefore, one aim of our present study was to see whether the TUNEL assay could be used to predict the more severe GVHD grades of III and above in a cohort of patients undergoing increased GVHD prophylaxis. There was a trend in favour of prediction of severe GVHD by TUNEL results when compared with GVHR alone, indicating that apoptosis might be used as an additional measure of GVH mediated damage in target tissues. However, despite the relative simplicity of the TUNEL assay, its overall positive correlation with GVHR ($p = 0.0001$), and the trend towards a positive correlation with clinical GVHD grade using the skin explant assay there was still a high number of false positive results. The major problem appears to be apparent non-specific staining. This resulted in a wide variation in staining patterns, which were observed in addition to the labelled apoptotic nuclei. In an attempt to reduce background staining and aid the morphological verification of apoptotic nuclei several steps in the assay were investigated. Quenching of endogenous peroxidase and increasing the concentration of

bovine serum albumin failed to reduce the background staining, which did not occur in the negative control when the TdT enzyme was omitted. Thus, it was concluded that the non-specific staining patterns were the result of factors other than TUNEL procedure itself.

Since Gravieli *et al* described the technique in 1992,¹⁴ several groups have reported the usefulness of the TUNEL assay.^{20–21} However, recently the TUNEL assay has come under some scrutiny, with an increasing number of research groups questioning the specificity and reliability of apoptosis detection using this assay. A group investigating apoptosis in rats with hepatomagen CPA induced hyperplasia of the liver reported the failure of the TUNEL assay to discriminate between apoptosis, necrosis, and autolytic cell death.²² This result has important implications when assessing apoptosis in graft versus host type reactions because tissue damage is also caused by necrosis.

“Recently the TUNEL assay has come under some scrutiny, with an increasing number of research groups questioning the specificity and reliability of apoptosis detection using this assay”

DNA fragmentation is common to different kinds of cell death and, in the liver—for example, apoptosis and necrosis *in vivo* occur asynchronously.²³ On the basis of these types of results the TUNEL assay may be unreliable for discriminating between different types of cell death, all of which involve DNA fragmentation. Although DNA fragmentation in apoptosis is more pronounced it may be necessary to accompany TUNEL staining with the demonstration of distinct morphological and ultrastructural features. There are also numerous publications describing a type of cell death resembling apoptosis, which may lack a characteristic apoptotic feature.^{24–25} Therefore, the application of more than one method of detecting apoptosis may be of more value than the use of a single method.

Furthermore, rather than detecting DNA breaks it may be possible to use another marker and other immunohistochemical staining methods to detect apoptosis. Monoclonal antibodies to FasL, TNF- α , granzyme B, and several caspase enzymes could all be used to detect the products associated with apoptosis. However, because the specific events leading to apoptosis in GVHD are unclear and likely to be complex, it may be necessary to adopt a panel, rather than using a single antibody.

In conclusion, the TUNEL assay correlated with apoptosis associated with GVHR in a significant number of GVHR grade II or above positive biopsies. In 18 samples where the GVHR and TUNEL assay could be correlated with clinical outcome there was an association between TUNEL results and clinical GVHD, but still a high number of false positive results. This may again reflect the fact that accurate *in vitro* prediction of GVHR is dependent on the type of GVHD prophylaxis. In this cohort of patients given cyclosporin plus methotrexate, the *in vitro* positive predictive results may have been altered by increased prophylaxis downregulating the observed clinical outcome.

Because the TUNEL assay may fail to discriminate sufficiently between various types of cell death the results could be improved by combining this assay with other methods of apoptosis detection.

Recently, the skin explant assay was used successfully to predict GVHD in a small cohort of paediatric matched unrelated transplants, where GVHD prophylaxis was in the form of cyclosporin and methotrexate alone.²⁶ In another European wide study of over 200 adult HLA matched sibling transplants a positive skin explant result has remained a risk factor for GVHD, irrespective of GVHD prophylaxis (AM Dickinson, personal communication, 2001). These last two studies

Take home messages

- The detection of apoptosis correlated with degree of graft versus host reaction using an *in vitro* assay and a higher degree of apoptosis tended to correlate with more severe graft versus host disease (GVHD), although there were a high number of false positives
- Further studies in a larger cohort of patients, using other methods to detect apoptosis in conjunction with the terminal deoxynucleotidyl transferase dUTP nick end labelling assay, may give additional insight into the complex immunopathophysiology of GVHD

emphasise the role that the skin explant assay can play in predicting GVHD and as an *in vitro* pretransplant GVHD predictive test. By further investigating the immunopathology of GVHD and the role of apoptosis, as shown by our present studies, a mechanism of early GVHD detection may be elucidated. As previously discussed, the detection of apoptosis in paraffin wax embedded tissue can be problematic; however, recent improvements to technology, such as double labelling protocols for TUNEL and active caspase 3, have meant that larger studies will be possible in the future. In addition, recent studies highlighting improvements in TUNEL, particularly at the permeabilisation stage, have reported an increase in the specificity of apoptosis detection.²⁷

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