Background and Objectives. The regulation of hematopoiesis by marrow stroma in vitro, has been shown to be abnormal in some patients with myelodysplastic syndromes (MDS). This study was performed to assess whether a range of mechanisms may be altered within the MDS microenvironment.

Design and Methods. The effects of diffusible factors produced by normal or MDS stromal layers on hematopoietic cells were studied by comparing the ability of media conditioned (CM) by normal or MDS stroma to regulate migration of target normal marrow CD34+ cells across 5 µm transmembranes. The ability of CM to stimulate hematopoietic cells was also assessed: changes in membrane polarity of KG-1a cells on exposure to stroma CM were compared. Subsequently, contact-mediated interactions between normal marrow CD34+ cells and normal and MDS stroma were studied: survival of allogeneic normal marrow CD34+ cells on live and glutaraldehyde-fixed normal and myelodysplastic stroma after 24h of coculture was measured using 7-aminoactinomycin D staining. To determine whether hematopoietic cell survival on normal and MDS stroma was related to oxidative stress within the stromal microenvironment, intracellular superoxide levels, both constitutively and induced by tumor necrosis factor-\(\alpha\) were measured within live stromal cells by FACScan analysis of ethidium bromide stained cells.

Results. The ability of CM from normal and MDS stroma to regulate short-term migration and activation of hematopoietic cells was similar. The mean percentage of apoptotic CD34+ cells (13±1%) adherent to glutaraldehyde-fixed myelodysplastic stroma was higher than on paired fixed normal stroma (11±1% (n=6, p=0.056). Constitutive mean levels of superoxide in myelodysplastic cultures (9.5±2.1) were greater than in normal stromal cultures (4.9±0.6; n=6). However, following treatment with tumor necrosis factor-\(\alpha\), the mean value for superoxide in myelodysplastic stromal cultures was unchanged (fractional change=0.99±0.56), compared with an increase in normal stroma (fractional change=1.6±0.1, p<0.05). No correlation was observed between superoxide levels, proportion of apoptotic CD34+ cells and percentage of CD14+ stromal cells [mean 8%, range 0-37% (myelodysplastic); mean 1.3%, range 0-5% (normal)].

Interpretation and Conclusions. Abnormalities of stromal function in myelodysplastic syndromes are likely to be heterogeneous in origin: altered matrix molecules and changes in superoxide within stromal cells may contribute to abnormal survival and development of hematopoietic cells within the myelodysplastic marrow microenvironment.

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Key words: stroma, diffusible factors, contact-mediated, superoxide, apoptosis

In myelodysplastic syndromes (MDS), blood cytopenias may arise from intrinsic changes in hematopoietic cells that increase the susceptibility to apoptosis.\(^1\) In addition, alterations in the functional properties of marrow stroma that could contribute to defective development of hematopoietic cells have been detected in a subset of patients.\(^2\) Thus, a variety of cellular interactions within the MDS marrow microenvironment may be abnormal, depending on the biological defect(s) within the hematopoietic and stromal compartments. While biological abnormalities within hematopoietic cells in MDS are believed to be heterogeneous, the possibility that various aspects of stromal function may be affected in different patients has not been extensively examined.
In the present study, the functional properties of stromal layers from the marrow of patients with MDS were compared with those from normal individuals. To determine whether diffusible factors released from normal or MDS stroma are different, the ability of medium conditioned by normal or MDS stromal layers to influence migration and activation of hematopoietic progenitors (HPC) was analyzed. Possible alterations in contact-mediated signaling by MDS stroma were examined by assessing survival of target hematopoietic cells on live or fixed normal and MDS stromal layers. Furthermore, factors with the ability to influence hematopoiesis, such as levels of intracellular superoxide (O2-) in stromal cells and the proportion of stromal monocytes/macrophages were also measured.

Design and Methods

Patients
Bone marrow samples from 10 normal individuals (age range, 59-74y) undergoing elective total hip replacement and 10 MDS patients (4 with refractory anemia (RA), 2 RA with ringed sideroblasts (RARS), 3 RA with excess blasts (RAEB) and 1 RARS that had transformed to acute myeloid leukemia (AML), age range, 59-82y) were obtained, after informed consent, for use in various experiments. The study was approved by the Tayside Committee on Medical Research Ethics. Mononuclear cells (MNC) were isolated and cryopreserved until use. Unique symbols were used to identify individual patients to facilitate comparison of data from different experiments.

Stromal cultures
Primary stromal layers were established in human long-term bone marrow culture (HLTBMC) medium (12.5% fetal calf serum, 12.5% horse serum, 4 mM L-glutamine, penicillin 100 U/mL, streptomycin 100 µg/mL, 2x10^-8 M hydrocortisone) in 50 mL flasks (Falcon) from 5x10^6 cryopreserved normal or MDS marrow MNC using the technique described by Moreau et al. A bi-weekly feeding schedule of cultures was followed and entire growth medium was replaced. Primary layers were trypsinized (0.25% trypsin, Sigma) once confluent (14-20 days for normal stroma), or if no developmental changes were evident after 7 days of culture, and secondary cultures were established in 35x10 mm dishes (Falcon). All studies on the functional properties of stroma, including co-culture experiments were performed in duplicate on secondary stromal layers. MDS stromal layers were studied in parallel with at least one randomly selected normal stromal culture to standardize experimental conditions.

Collection of stroma conditioned medium
HLTBMC medium conditioned by normal and MDS stromal layers was aspirated after 5 days of medium replacement, filtered through a 0.2 mm low adsorption surfactant-free cellulose acetate membrane filter (Sartorius) and stored at -70°C as conditioned medium (CM).

Development of normal and MDS stromal layers
Stromal morphology. Stromal layers were examined bi-weekly under a phase-contrast microscope to identify structural differences (if any) between MDS and control stroma. The two main features under scrutiny were the ability to attain confluence and to produce adipocytes. Confluence of stroma was classified as being of grade I (>80% of growth surface-area covered), grade II (patchy confluence) and grade III (non-confluent). Stromal ability to produce fat cells was characterized by the absence (-) or presence of foci of adipocytes. Poorly adipogenic stroma contained fewer than 3 foci (+); 3 or more foci of adipocytes were detected in adipogenic stroma (+/+).

Proportion of monocytes/macrophages (CD14+) in stromal layers. Series of 1x10^5 trypsinized cells from normal and MDS secondary stromal cultures were incubated with 5 µL of dual color reagent (DAKO), containing FITC conjugated anti-CD45+ and PE conjugated anti-CD14+ antibodies and fixed in 1% paraformaldehyde. On FACS analysis, monocytes/macrophages were identified as CD45^high CD14^high events in dot-scattergrams combining FL1 and FL2.

Functional properties of normal and MDS stromal layers
Comparison of diffusible factors within stroma CM. Ability of CM to induce transmembrane migration of HPC. The ability of CM from normal and MDS stroma to mobilize purified marrow CD34+ cells was tested in transmigration assays. In paired studies, numbers of CD34+ cells that migrated across a 5 µm polycarbonate membrane towards a gradient of normal and MDS CM were counted after 3.5h. A purified population of CD34+ cells (96.2-99.7% on FACScan analysis) was obtained from normal marrow MNC, using the MiniM ACS Cell Isolation kit (Miltenyi Biotec Inc.). Six hundred microliters of CM from normal, MDS stroma or HLTBMC medium (control) were added to individual 6.5 mm cluster-wells (Corning-Costar). Transwells were inserted and 75x10^3 CD34+ cells were introduced inside the tran-
swells (final volume 100 µL). After incubating at 37°C in 10% CO₂ and 5% O₂ for 3.5h, insertions were removed and numbers of cells that had transmigrated into CM and control medium were enumerated on a FACSscan (Becton-Dickinson). Flow-cytometric counts were obtained for each sample for a constant time of 200 seconds. The ability of CM from normal and MDS stroma to influence migration was calculated from the migration index (ratio between cells that migrated in response to CM and cells that migrated to control media). To standardize the target cell-type in migration experiments, similar studies were also performed using 1.5×10⁵ KG-1α cells (obtained from ECACC) as model HPC instead of marrow CD34⁺ cells from different donors.

Ability of CM to activate KG-1a cells. To investigate whether early responses of KG-1a cells to CM, occurring before those detected by migration assays, could be used to identify differences between normal and MDS CM, changes in membrane potential (δψ) of KG-1a cell membranes were compared after exposure to CM. The KG-1a cells (1.5×10⁶) were suspended in 1 mL of CM from normal or MDS stroma or in control HLTBMC medium. 3,3’-dihexyloxacarbocyanine iodide [DiOC₆ (3)] (Molecular Probes), a dye that indicates δψ, was added to a final concentration of 40 nM. Cells were incubated at 37°C in the dark for 5 min and then analyzed immediately on the FACSscan. Cell suspensions incubated in CM without the addition of dye were used as negative controls. Data on 10,000 events measuring changes in DiOC₆ (3) fluorescence (FL1) were acquired and analyzed using Cell Quest software (Becton-Dickinson).

Comparison of stromal regulation of adherent hematopoietic cell survival

Apoptosis of CD34⁺ cells in contact with live stromal layers. The stromal layers were co-cultured with 1×10⁶ plastic non-adherent MNC (NAMNC) (non-adherent cells recovered after 60 minutes of incubation of mononuclear cells in 15% FCS at 37°C) from normal marrow. After 24h, adherent layers were trypsinized, and the proportion of live and apoptotic CD34⁺ cells, quantified by 7-amino-actinomycin D (7-AAD) (Sigma), staining in a FACScan assay. In this assay, live cells exclude 7-AAD (7-AADnegative), while early and late apoptotic (necrotic) cells are more permeable to 7-AAD (7-AADdim and 7-AADbright, respectively). Briefly, 5×10⁵ trypsinized cells stained with FITC-conjugated anti-CD34 and PE-conjugated anti-CD34 antibodies were re-suspended in a staining solution containing 20 µg/mL of 7-AAD for 20 min at 4°C. Cells were then fixed in 500 µL 2% paraformaldehyde solution, containing 20 µg/mL of actinomycin D (Sigma). Unstained fixed cells, previously incubated in FITC and PE-conjugated mouse IgG antibodies were used as the negative control.

Data on 20,000 CD45⁺ events were acquired for assessing CD34 positivity. In dot-scattergrams combining FL2 and SSC, CD34⁺ cells were identified as events with high FL2 and low SSC (R1). The fluorescence intensity in FL3 (7-AAD) for cells within R1 was analyzed in scattergrams of FSC versus FL3. Gates were drawn around cells which were FL3negative (R2, live) and FL3dim+bright (R3, apoptotic) and the proportion of cells within each gate was calculated. Apoptosis of CD34⁺ cells in contact with metabolically inactive stromal layers. To examine the functional properties of physical components (matrix and adhesion molecules), normal and MDS stromal layers were rendered metabolically inactive by treating adherent layers with 2% glutaraldehyde. After co-culturing 1×10⁶ NAM NC with fixed stroma for 24h, adherent hematopoietic cells layers were trypsinized and stained with antibodies against CD45 and CD34, conjugated with FITC and PE respectively (Becton-Dickinson) and 7-AAD. Triple-color analysis on FACSscan was used to quantify apoptosis in CD34⁺ cells adherent to fixed normal and MDS stromal layers.

Superoxide (O₂⁻) levels in stromal cells

To determine whether the MDS microenvironment is more pro-oxidant than normal, levels of O₂⁻ within normal and MDS stromal cells were measured, using a technique similar to that described by Narayan et al. In brief, spent medium in stromal cultures was replaced with fresh HLTBMC C. Cultures were incubated at 37°C in 10% CO₂ and 5% O₂ for 30 minutes, following which stromal cells were trypsinized. Single stromal cells were resuspended in culture medium removed prior to treatment with trypsin and incubated for an additional 30 minutes in the presence of 10 μM hydroethidine (Molecular Probes) in dimethylsulfoxide (DM SO). Controls received an equal volume of DM SO. Thereafter, cells were placed on ice and fluorescence from 10,000 stromal cells was collected using a 585/42-nm band filter on the FACSscan. Values of mean fluorescence in tubes stained with hydroethidine were used as an index of intracellular O₂⁻ levels after subtracting fluorescence from control tubes (Cell Quest). Intracellular stromal O₂⁻ was also measured after treating normal and MDS cultures with 25 ng/mL TNF-α for 30 min.
Statistical analysis

All experiments were performed in duplicate and the results were expressed as the average of two values. The arithmetic mean was used to measure the central tendency of data from normal and MDS cultures, and the dispersion of values around the mean was expressed as the standard deviation (s.d.) or standard error of the mean (s.e.m.). In co-culture experiments studying apoptosis, results from MDS co-cultures were expressed as a fraction of the results from paired normal stroma. The significance of changes \( (p < 0.05) \) between results from studies on normal or MDS stromal layers was measured using Student's paired t-test. Cell Quest (Becton-Dickinson) was used to analyze data generated on the FACScan.

Results

Development of stromal layers

Stromal morphology. Stromal layers from normal marrow attained grade I confluence and formed >3 foci of adipocytes. In contrast, the morphology of MDS stroma was heterogeneous. The symbols may be used to identify individual patients in subsequent Figures and Tables (Table 1).

Proportion of CD14+ cells in stroma. A wide variation in the proportion of monocytes within stromal layers was observed: the mean percentage in normal stroma was 1.3% (range = 0.4-9.5%, n=6), whereas a mean of 8% (range = 0-36.7%, n=6) of cells in MDS stroma were monocytes (Table 1).

Functional properties of normal and MDS stromal layers

Diffusible factors within stroma CM. Ability of CM to induce transmembrane migration of HPC. Paired studies examined the effects of CM from normal and MDS stroma on migration of purified CD34+ cells across a 5 µm transwell after 3.5 hours. Cell numbers in wells containing CM from normal or MDS stroma were expressed as a percentage of those in wells with control medium (n = 6). While greater numbers of cells migrated to wells containing normal (p < 0.05) or MDS (p < 0.05) CM than control medium (Figure 1), the mean percentage increase in the number of cells migrating in the presence of normal CM (604±426) was, however, not significantly different from that seen in response to MDS stroma CM (698±594). Similarly, in studies using KG-1a cells as target population, the mean numbers of KG-1a cells migrating in response to normal CM (21,611±6,282) and MDS CM (22,185±7,517) were not different (n = 6).

Ability of CM to activate KG-1a cells. Metabolic changes in KG-1a cells after 5 minutes of exposure to CM were studied by monitoring \( \delta \psi \) of KG-1a cells using the cyanine dye DiOC6.3 In histograms showing fluorescence (FL1) from cells incubated in the presence of control medium, a marker (M1) was set to include >90% of cells (steady-state \( \delta \psi \)) (Figure 2). A second marker (M2) was drawn to identify events with lower intensity of fluorescence, i.e. lower \( \delta \psi \). Events falling within this marker were designated to represent cells with a depolarized membrane. After 5 minutes of incubation in control medium, a mean of 8% (range 5-10%, n = 4) of cells had depolarized membranes, whereas a mean of 81% (range 50-97%) of cells in normal CM and 79% (range 41-98%) in MDS CM had decreased \( \delta \psi \). While exposure to CM resulted in a significant increase in depolarized events compared to control medium, the ability of CM from normal and MDS stroma to induce changes in \( \delta \psi \) was similar.

Comparison of stromal regulation of adherent hematopoietic cell survival.

Apoptosis of CD34+ cells in contact with live stromal layers. In co-cultures of normal NAMNC with live normal stroma (n = 6), a mean of 11±9.1% of CD34+ cells adherent to stroma after 24h was apoptotic. The mean percentage of apoptotic CD34+ cells on MDS stroma was 17.7±15.1%. However, higher levels of apoptosis were detected in 2/6 co-cultures with MDS stroma (Figure 3).
Apoptosis of CD34+ cells in contact with metabolically inactive stromal layers. The mean percentage of apoptotic CD34+ cells on fixed normal stroma was 11±9.8%. Apoptotic cell numbers on fixed MDS stroma were marginally higher than on corresponding normal stroma and the mean percentage of apoptotic cells was 13.2±10.7% (p = 0.056).

O2− levels in stromal cells

In ethidium bromide based FACScan assays, the fluorescence from normal stromal cultures (constitutive) was 4.9±0.6 (mean±s.e.m., range = 3.8-7.6, n=6). Corresponding MDS stromal cultures had a higher mean fluorescence (9.5±2.1, range = 4.2-16.5), with values from some patients reflecting high constitutive levels of O2− (Figures 4 and 5). Differences between overall means of normal and MDS stromal cultures were however, not statistically significant.

Following exposure to 25 ng of TNF−α, significant increases in intracellular O2− occurred in normal stromal cells (Figures 4 and 5.1): the mean fluorescence (7.8±0.8) in TNF−α treated stroma represented an increase of 1.6±0.1 over the corresponding value from untreated cultures (4.9±0.6, p = 0.003). A heterogeneous response was, however, observed when

MDS cultures were treated with TNF−α. Mean fluorescence increased in only 2/6 MDS stromal cultures, whereas it decreased in 4/6 (Figure 4 and 5.2). As a result the mean fluorescence after treating MDS stroma with TNF−α (8.3±1.4) was not significantly different from that of corresponding control cultures.

Discussion

Multiple aspects of marrow stromal function were assessed in this study, to determine whether
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effects of diffusible factors (in CM) and contact-mediated signals from MDS stroma on HPC kinetics were different from those of normal stroma. Regulation of short-term kinetics of hematopoietic cells by diffusible stromal factors appears to be unaffected in MDS: migration of target CD34+ and KG-1a cells in response to gradients of normal or MDS CM was similar. Furthermore, when early cellular responses to CM were studied, a similar decrease in δψ of KG-1a cells (suggesting cellular activation) was observed on exposure to both normal and MDS CM. Although decreased fluorescence intensity of DiOC6(3) in cells can indicate early apoptosis, the reduction in δψ in KG-1a cells on exposure to stroma CM was unlikely to be due to onset of cell death: externalization of phosphatidylserine on the plasma membrane (a feature of apoptotic cells) was not detected, even after 3.5 hours of incubation of KG-1a cells in CM (data not shown).

In studies on contact-mediated stromal function, the survival of CD34+ (within target normal NAM-
In an attempt to identify mechanisms that may deregulate development of CD34+ cells on live MDS stromal cultures, intracellular O$_2^-$ levels within normal and MDS stromal cells were compared under basal conditions, as well as after induction with TNF-α. Though the mean levels of intracellular O$_2^-$ detected constitutively in MDS stroma were almost double that in normal stromal cells, the mean difference was not statistically significant due to heterogeneity of O$_2^-$ levels between stroma from different MDS patients. Interestingly, a higher proportion of apoptotic CD34+ cells was detected in the two MDS stromal cultures (c and o) with high constitutive levels of O$_2^-$.

In contrast to the predictable increase in O$_2^-$ levels in normal stromal cells after treatment with TNF-α, the response in MDS cells was heterogeneous. The oxidative response of MDS stroma may thus be different from that of normal stroma. Though a high percentage of CD14+ cells (monocytes/macrophages) was detected in 2/6 MDS stromal layers (both from patients with RAEB), numerical changes in macrophages did not appear to contribute to either the alteration in O$_2^-$ levels or survival of donor hematopoietic cells.

These preliminary experiments thus suggest that a variety of stromal abnormalities may be present in different patients with MDS, and warrant further studies in larger numbers of patients. In particular, examination of functional properties of stromal matrix molecules, and changes in reactive oxygen species within the marrow may help to identify mechanisms that contribute to pancytopenia in some patients and facilitate evolution to AML in others.

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ST, MJP: conception and design; MJP, ST, MDH, DTB: drafting and revisions; MJP: final approval.

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Potential implications for clinical practice
Should further studies using in vitro models described in this study suggest a correlation with the clinical profile of MDS in individual patients, it may be possible to develop in vitro assays that identify high-risk disease.

References


