Effect of Tissue Shipping on Plasma Cell Isolation, Viability, and RNA Integrity in the Context of a Centralized Good Laboratory Practice–Certified Tissue Banking Facility

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Abstract

The Multiple Myeloma Research Consortium has established a tissue bank for the deposition of bone marrow samples from patients with multiple myeloma to be mailed and processed under good laboratory practices. To date, over 1,000 samples have been collected. At this time, limited information is available on shipped bone marrow aspirates in regards to cell viability, yield, purity, and subsequent RNA yield and quality. To test these determinants, we did a pilot study on behalf of the Multiple Myeloma Research Consortium where samples were drawn at Mayo Clinic Rochester (MCR) pooled and split into two equal aliquots. One-half of each sample was processed following good laboratory practices compliant standard operating procedures, immediately after sample procurement, at MCR. The CD138+ cells were stored at -80°C as a Trizol lysate. The other half of the aspirate was sent overnight to Mayo Clinic Scottsdale where they were processed using identical standard operating procedures. The RNA was extracted and analyzed in a single batch at MCR. At both locations, samples were assayed for the following quality determinants: Viability was assessed using a three-color flow cytometric method (CD45, CD38, and 7-AAD). Cell counts were done to determine plasma cell recovery and post-sort purity determined by means of a slide-based immunofluorescent assay. RNA recovery and integrity was assessed using the Agilent Bioanalyzer. Lastly, gene expression profiles were compared to determine the signature emanating from the shipment of samples. Despite minor differences, our results suggest that shipment of samples did not significantly affect these quality determinants in aggregate. (Cancer Epidemiol Biomarkers Prev 2008;17(3):666–73)

Introduction

In modern clinical drug development, validating the efficacy of therapeutic agents entails conducting randomized trials. These trials require the participation of a large number of patients to ensure adequate statistical power for meaningful conclusions. As such, they are usually conducted in the setting of multicenter, and sometimes international, cooperative groups. Additionally, the need for obtaining high-quality purified tumor cells for ancillary research studies from these trials is ever increasing. To this end, the Multiple Myeloma Research Consortium has established a tissue bank to collect bone marrow from patients with multiple myeloma. To assure the quality of these samples, we have implemented standard operating procedures for the collection, isolation, and storage of the tumor cells as well as striving toward good laboratory practices standards. This has led to an internal quality assurance unit and an information technology infrastructure for the registration, the sample tracking, and the sample attributes.

Unlike many other hematologic malignancies, the tumor cells from multiple myeloma patients coexist with many normal hematopoietic cells in the bone marrow and thus need to be purified using some type of selection method. Our experience with shipped samples within the context of the Eastern Cooperative Oncology Group has shown the ability to obtain good plasma cell recoveries from mailed bone marrow aspirates. Using these samples, we have shown the use of slides for fluorescence in situ hybridization (1, 2), DNA for mutation analysis (3), and RNA for gene expression profiling (GEF; ref. 4). However, these studies did not compare results with a split sample being processed immediately. The RNA in particular was of the most concern due to its instability. To directly address this question, a pilot project was set up to compare the isolation of plasma cells immediately after bone marrow aspiration versus having the aspirate shipped priority.

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overnight at 4°C and processed the following day with particular focus on the RNA and the subsequent expression profile.

Materials and Methods

Samples. Bone marrow aspirates were obtained after informed written consent and collected into tubes containing ACD as the anticoagulant. The bone marrow was pooled and gently mixed to ensure uniform cell distribution.

To limit the number of variables in this study and yet mimic mailing conditions, we decided to use one other site, Mayo Clinic Rochester (MCR). MCR was supplied with and used the same standard operating procedures as Mayo Clinic Arizona (MCA) and has years of experience with these plasma cell separation methods. We designed the study the following way: MCR pooled the bone marrow aspirate (up to 40 mL), split in half, processed one-half of it immediately, and shipped the other half to the Multiple Myeloma Research Consortium tissue bank located at MCA. Post-plasma cell purification samples were placed in Trizol and frozen and the MCA samples were sent back to MCR on dry ice. MCR then extracted the RNA from the Trizol and did both quantitative and integrity analysis. This analysis included the total RNA recovered as determined by spectrophotometer readings and the quality of the RNA determined by the 28S/18S ratio ascertained from the Agilent Bioanalyzer. GEP was done on all samples where sufficient RNA quantities and quality were available. Slides from the CD138+ selected cells were also sent to MCR for plasma cell purity analysis. This was done to avoid possible variability among readers. All other quantitative and qualitative determinants were done at the respective sites, which included the following: recovery of CD138+ plasma cells as well as viability of both the whole bone marrow and the CD138 selected fractions (Fig. 1).

Flow Cytometry. The percentage of live, apoptotic, and dead cells were determined on ammonium chloride-lysed whole bone marrow and CD138+ cell fraction by flow cytometry. These variables were determined using a three-color apoptosis assay as described previously (5). Briefly, cells were stained using CD45 conjugated to FITC (CD45-FITC; Becton Dickinson) and CD38 conjugated with phycoerythrin (CD38-PE; Becton Dickinson) to identify the plasma cells (45-/dim38++) and 7-AAD to identify the apoptotic/dead fractions. All samples were run using the BD FACScan flow cytometer.
and the data were analyzed using the Cell Quest software program (Becton Dickinson). Regions were drawn to identify the percentage of cells in each of the three possible populations: alive, dead, or apoptotic. Plasma cells that were negative for 7-AAD were considered alive as the membranes were intact enough to exclude the dye; cells that were bright 7-AAD positive were considered dead and very permeable to the dye; cells undergoing apoptosis had 7-AAD staining between these two values. The percentage of each fraction was calculated by the software program.

**Plasma Cell Isolation.** Plasma cells were isolated from the whole bone marrow using the immunomagnetic bead selection. We used a monoclonal mouse anti-human CD138⁺ antibody microbeads and the AutoMACS cell separator (Miltenyi Biotech). The RBCs were lysed using an ammonium chloride lysing procedure, and cell counts were done using a Coulter counter. Antibody bead conjugates were incubated and the cells were washed using PBS containing 2% bovine serum albumin and 1 mmol/L EDTA (bead buffer). Cells were resuspended in 4 mL bead buffer and the sample was loaded onto the AutoMACS. We used POSSELDS on all separations. This program uses two columns, which increases the plasma cell purity at the expense of some plasma cell loss. The cells from the POS 2 port were removed, counted, aliquoted for flow cytometry, and the remainder placed in Trizol at a concentration no greater than 10 million/mL and frozen at -80°C until all samples were collected.

**Post-Sort Purity Check.** The purity of all sorts was confirmed using a three-color immunofluorescent slide-based method. Approximately 10,000 cells were removed from the positive fraction after sorting and spun onto a slide using a cytospin centrifuge. The slides were allowed to air dry. A circle was drawn around the cells using a Super PAP Pen (The Binding Site), dried, and placed into a coplin jar containing 95% ethanol for 5 min. The slides were removed, dried, and placed into a new coplin jar with APK wash solution (Ventana Medical Systems). The slides were removed and dried. Antibody mix (100 μL) containing 10 μL anti-κ-AMCA, 10 μL anti-λ-FITC, and 80 μL RPMI containing 10% FCS was added to each slide and incubated in the dark for 30 min at room temp. The slides were washed three times (3 min/wash) by placing the slides in a coplin jar with APK and gentle agitation. After the last wash, the slides were air dried, 10 μL Antifade with propidium iodide (Vector Laboratories) was added, coverslip was added, and 100 cells were scored using a fluorescent microscope and a triple-pass filter. The percentage of FITC-positive, AMCA-positive, and propidium iodide–positive only cells are recorded and checked against the known isotype to ensure quality of the sort.

**RNA Purification and Integrity Assessment.** RNA was isolated from the Trizol using a chloroform extraction protocol (6). Briefly, Trizol samples were homogenized using a 20-gauge needle, chloroform was added, and the tubes were centrifuged. The aqueous phase, containing the RNA, was removed and isopropyl alcohol was added to precipitate the RNA. The RNA pellet was washed with 75% ethanol and the pellet was allowed to dry. The dried RNA pellet was then suspended in RNase-free water. The RNA was further “cleaned up” using the Qiagen RNeasy columns. The concentration of the RNA was determined by using a ratio of the nucleic acid absorbance at 260 nm ($A_{260}$) to

**Figure 2.** Comparison between (A) apoptosis, (B) plasma cell purity, (C) plasma cells recovered, and (D) RNA recovered per million plasma cells in samples processed immediately at MCR and those shipped and processed 24 h later in Mayo Clinic Scottsdale.
the protein with the absorbance at 280 nm ($A_{280}$) on the spectrophotometer. Additionally, the RNA integrity was assessed on the Agilent 2100 Bioanalyzer (Agilent Technologies) using the 28S/18S ratio. High-quality total RNA samples have distinct features that include the 18S and 28S ribosomal peaks. The 28S/18S ratio should be >1.0 for successful GEP. Also, the baseline between 29 s and the 18S ribosome should be relatively flat and free of small round peaks. Lastly, the baseline between the two ribosomal peaks should not have well-defined peaks.

**Gene Expression Profiling.** Gene expression analysis was done on RNA from CD138<sup>+</sup> selected plasma cells using the Affymetrix U133A chip (Affymetrix). Microarray hybridization has been reported previously (7). Probe level data were normalized using the Affymetrix Microarray Suite 5.0 algorithm. Gene expression intensity values were log transformed, median centered, and analyzed using GeneSpring 7.3.1 GX (Agilent Technologies). For quality of the gene expression data, we assessed the GAPDH $3'/5'$ ratio and the actin $3'/5'$ ratio. Samples with poor quality results were identified if the ratios were >1.25 and >3.0, respectively. The comparability of the gene expression data of the paired samples was assessed in several ways. First, the raw expression values of the pairs were plotted on an X-Y plot to check for concordance. Second, the samples were clustered in an unsupervised manner using genes that varied across the individual samples.

These genes were first identified by Welch’s ANOVA using variance computed by applying the cross-gene error model based on deviation from 1 available within GeneSpring. This overcomes the lack of replicates and variance associated with the individual samples and is similar in principle to variance filtering. Unsupervised clustering was done using the hierarchical agglomerative algorithm. Pearson’s correlation coefficient and centroid linkage were used as similarity and linkage methods, respectively. To detect possible differences between samples processed immediately and those that were shipped, we extracted genes that had 1.5-fold difference in expression and were statistically significant at a corrected $P$ value of 0.05 by Student’s t test with Benjamini-Hochberg multiple testing corrections. These differentially expressed genes were then assessed for Gene Ontology (GO) enrichment using GeneSpring.

**Results**

Despite some variability, there were no consistent or significant differences in the quality determinants when the two sets of samples were compared (Fig. 2; Table 1). The cell viability, as determined by a three-color flow cytometric assay, was evaluated in 9 of 14 paired samples and was similar in both sets of samples (Fig. 2A). The majority of the cases showed equivalent percentages of cells in the apoptotic/dead fraction. In 3 of 9 samples, the apoptotic fraction was higher in the mailed samples, whereas 5 of 9 samples from the immediately processed samples were higher and 1 of 9 were equivalent. The median apoptotic fraction for the immediately processed sample was 17.4% (range, 2.1-29.1%) compared with the mailed samples with a median of 8.0% (range, 3.7-62.8%; $P = 1.0$). The whole bone marrow samples showed a similar pattern of apoptotic/dead cells as the plasma cell fraction (data not shown). We were able to collect highly enriched plasma cell population (Fig. 2B). The mean percentage of purity was 95.5% (range, 75-100%) for the immediately processed samples and 89% (range, 56-99%) for the shipped samples ($P = 0.05$).

In the majority of the samples, the percent plasma cells were equivalent. There were 5 samples that showed some discrepancy. Of the immediately processed samples, 3 of these had better purity, whereas 1 of the 12 mailed samples showed a higher purity. The plasma cell yield was slightly lower in the shipped samples (Fig. 2C) with a median of 4.3 million on the immediately processed samples compared with 2.2 million on the mailed samples. The difference in recoveries may be possibly due to some shipment-associated apoptosis and subsequent loss of cell surface CD138 antigen (8). However, as mentioned, the purity of the samples shipped was very similar to that of the locally processed ones. Subjective analysis of the RNA was similar between both groups. The median RNA recovery normalized to μg/million cells was 5 μg (range, 2.1-9.7) for the MCR processed samples and 4.6 μg (range, 0.8-10.3) for the Mayo Clinic Scottsdale samples ($P = 0.33$; Fig. 2D). There was no evidence of additional degradation in the shipped samples after purification when the 28S/18S ratios were compared (data not shown). Adequate RNA, a minimum of 3 μg total RNA at a concentration of at least 0.757 μg/μL and a 28S/18S ratio greater than 1.4, was available for GEP studies in 7 paired samples. The other 7 pairs failed our quality control metrics due to either degraded RNA (5 pairs) or an insufficient amount of RNA (2 pairs). All 14 GEP results passed quality control as assessed by GAPDH and actin $3'/5'$ ratios (minimum values of 1.25 and 3.0, respectively).

The concordance in the GEP results between the paired samples is also extremely good. Five of the pairs showed >99% of genes with expression within 2-fold of each other. On unsupervised clustering, all the paired samples were clustered next to each other with very similar expression profiles, although in several pairs a small cluster of genes were overexpressed in the samples that were processed 24 h after aspiration (Fig. 3A).

<table>
<thead>
<tr>
<th>Variables</th>
<th>n</th>
<th>Rochester, median (range)</th>
<th>Scottsdale, median (range)</th>
<th>P (Mann-Whitney U test)</th>
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<tbody>
<tr>
<td>Recovered plasma cells</td>
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<td>4.3 (1.2-19.2)</td>
<td>2.2 (0.8-12)</td>
<td>0.11</td>
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<td>% Plasma cells</td>
<td>12</td>
<td>95.5 (75-100)</td>
<td>89 (56-99)</td>
<td>0.05</td>
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<tr>
<td>% Apoptosis</td>
<td>9</td>
<td>17.4 (2.1-29.1)</td>
<td>8.0 (0.6-22.8)</td>
<td>0.4</td>
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<tr>
<td>Cell count 138 Trizol</td>
<td>14</td>
<td>3.6 (1.5-6.4)</td>
<td>1.9 (0.6-5)</td>
<td>0.13</td>
</tr>
<tr>
<td>RNA yield, μg/million</td>
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<td>5.0 (2.1-9.7)</td>
<td>4.6 (0.8-10.3)</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Table 1. Comparison of variables between samples processed immediately at Rochester and those shipped to Scottsdale
No differentially expressed genes between samples processed immediately and after 24 h passed our statistical filter, but this could be attributed to relatively low sample size Table 2. Eighty-two probe sets have a 1.5-fold difference in expression level between those samples processed immediately and 24 h later. Fifty-one probe sets were up-regulated and 31 down-regulated in the samples processed at 24 h (Fig. 3B). Interestingly, these genes are enriched for GO processes that represent cellular response to environmental stress (Table 1).

Discussion

When establishing a biorepository, one must not only meet the needs of the immediate studies at hand but also have the foresight for what may need to be stored for projects at a much later time. There are many variables one must consider in establishing a tissue bank. Some of these include the harvest of the tumor, sample processing, and storage of the samples. We have shown previously that we were able to use slides from mailed tissue for immunohistochemistry/immunofluorescence as well as fluorescence in situ hybridization studies. Decisions about what storage media to use for future isolation of RNA, DNA, and protein can also be problematic. We chose for our tissue bank to store the tumor cells in Trizol reagent. This has allowed us to obtain high-quality RNA, even in shipped samples, as shown here. We were also able to obtain high-quality DNA for array comparative genomic hybridization (9) and sequencing with very good results. Others have
Figure 3 Continued. B, 82 probe sets were differentially expressed between samples processed immediately (Rochester) and 24 h later after shipping (Scottsdale).
shown previously the use of protein from Trizol extractions for Western blots (10).

There are not many studies published that have looked at RNA stability and shipment of cells isolated from bone marrow. These issues were highlighted in a recent pharmacogenomics study involving patients entered into multicenter international trials for bortezomib, a recent Food and Drug Administration–approved treatment for multiple myeloma (11). There was significant attrition at various steps along the way from consenting patients, obtaining samples to various quality control measures (RNA quality, microarray hybridization quality, and contamination issues) such that, on average, only 34% of collected samples have analyzable gene expression data. In these studies, samples are collected and processed (isolation of malignant cell of interest by negative selection) at the individual collection sites before being shipped to a central laboratory for gene expression studies. Of note, in their study, the greatest attrition occurs at the step of RNA quality (range, 37-65%). Furthermore, this attrition appears worse in the international studies compared with the US-only studies (62% versus 39% attrition). This suggests that one of the main reasons for not being able to obtain good-quality RNA may relate to heterogeneity in sample collection and processing procedure or the need to transport samples. These issues are extremely important in the current age of large-scale multicenter and often international drug trials.

In this study, we focused on the feasibility of using a single centralized processing and storage center versus multiple local centers in regards to the quality of the overall specimen with emphasis on the RNA as transcriptional profiling is integral to pharmacogenomics study toward individualized therapy. The ability to use a centralized bank would have significant cost savings and quality assurance issues would be much more manageable. However, all of these advantages would quickly be diminished if the quantity and quality of the tumor were such that it rendered it unusable. We therefore wanted to compare the quality of the RNA from the tumor cells both before and after shipping to assess the effect on GEP. In doing so, we investigated the viability and recovery of the plasma cells and the quantity as well as quality of the RNA. We did not see any consistent differences in any of these variables between samples processed within a few hours of the aspirate to those shipped at 4°C overnight.

When assessing the pairs, >90% of genes have expression within 1.5-fold of each other. In fact, only 1% of probe sets have, on average, >2-fold differences in expression between samples processed immediately and shipped. Our results are in contrast with a previous publication (12), which found that only 8.5% of probes never exceeded 1.5-fold in all experiments and 38.4% of probes have >2-fold difference in expression. One major difference is that, in our study, only RNA from CD138+ malignant plasma cells, as opposed to unselected bone marrow cells, are isolated for gene expression studies. It is conceivable that transcriptional programs of plasma cells or malignant cells are less prone to fluctuations or environmental changes. However, despite having a much lower number of genes with significant changes in gene expression, the genes whose expression does change are mainly involved in stress response as corroborated by the GO analysis. This result is similar to that reported in a previous study. More importantly, the gene expression profile was not altered in a biologically relevant manner as the paired samples
essentially cluster together and also express genes relevant to their biology in similar manner such that their molecular classification is not affected.

Further analysis of RNA recoveries revealed that samples where we recovered <2 million plasma cells rendered less than ideal amounts of RNA and the quality of this RNA was more likely to be degraded. This is shown in Fig. 2D where the greatest differences (µg/million PC) is observed in sample pairs 8 to 14. Samples 8 to 14 started with fewer plasma cells in Trizol than samples 1 to 7 [median, 1.5 million (range, 0.8-9.2) versus 7.4 million (range, 2.0-19.2)]. Additionally, the RNA was degraded in 5 of 8 of these samples. From these observations and additional testing, we have determined samples yielding <2 million plasma cells are less likely to yield enough high-quality RNA for expression profiling (regardless of whether or not they are processed immediately). Within the context of the Multiple Myeloma Research Consortium biobank, we have had very good results with samples where plasma cell recoveries were >2 million plasma cells. To date, we have extracted quality RNA/DNA with successful profiles in ~85% of these cases.

Biobanking is becoming an important tool for assisting in the categorization of disease, prognostic markers, as well as potential drug discovery leading to personalized medical care. Studies such as this should be done to insure the quality of what is put into a bank. The Multiple Myeloma Research Consortium tissue bank collects and stores peripheral blood mononuclear cells, plasma from both the blood and the bone marrow, ammonium chloride-lysed whole bone marrow, cyto-spin slides, and CD138⁺ and CD138⁻ cells. To ensure the Multiple Myeloma Research Consortium would have the best tissue bank for multiple myeloma, we have put together ~70 standardized operating procedures for the shipment, receipt, isolation, and storage of these samples. In this study, we affirmed that a centralized bank could obtain and isolate tumor cells without a substantial reduction in RNA quality or GEP outcome.

References
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