cytokine responses induced by 2 immunodominant peptides: Jkα1 and Jkα2 (Figure 1). There were 2 populations of anti-Jkα alloimmunized donors individualized: 4 donors with a Th1 (IL-2/IL-4) response, and 7 donors with a Th2 (IL-4/IL-2) response. In response to the same peptide, 2 different donors may produce different cytokines (IL-2 or IL-4). As a control of peptide-specific response, the nonalloimmunized population (7 donors) did not produce significant levels of cytokines (data not shown).

This Th1/Th2 dichotomy was not due to delays in kinetics of IL-2 and IL-4 productions, whatever the poststimulation time tested (3, 6, 20 hours; data not shown). Nor was it related to particular donor DRB1* or DQB1* molecules. On the contrary, the frequencies of DRB1*01 (82%) and DQB1*05 (100%) phenotypes observed in these alloimmunized donors producing anti-Jkα, without other alloantibodies, were higher compared with the expected phenotypic frequencies in the white population, 18.1% and 20.8%, respectively. This observation raises the question as to whether these molecules are associated with genetic susceptibility to Jkα alloimmunization, as previously demonstrated for DRB3*0101 and alloimmunization against platelet-specific antigen HPA-1a. The Jkα1/Jkα2 immunodominance in this population may be explained by the high affinity of both peptides for DRB1*01 as indicated by the HLA-peptide binding motif predictions (data not shown). Because DQB1*05 binding prediction data are not available, we cannot exclude that DQB1*05 increased frequency is the consequence of the known linkage disequilibrium with DRB1*01. Jkα1 and Jkα2 peptide presentation by HLA-class II molecules are currently being investigated.

In conclusion, our data suggest that (1) a clear dichotomy (IL-2/IL-4) exists among anti-Jkα alloimmunized donors in response to specific Jkα peptide stimulations; (2) the response induced by one peptide may vary (IL-2/IL-4) depending on the donor tested; and (3) as expected from peptide binding motif predictions, the Jkα protein contains limited dominant T-helper epitopes, and the DRB1*01 molecule could be implicated in the Jkα peptide presentation.

A better knowledge of cellular alloimmunization against Jkα antigen could be extended to other RBC antigens. Identification of such immunodominant peptides, the cytokine patterns induced, and the HLA class II molecules implicated in their presentation would facilitate the design of new therapeutic strategies including the specific control of alloimmunization with peptide antigen tolerogens or the ex vivo induction of regulatory T cells.

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References

To the editor:

Chondrogenic differentiation of mesenchymal stem cells is inhibited after magnetic labeling with ferumoxides

Magnetic resonance (MR) tracking of magnetically labeled cells is a relatively new technique to noninvasively determine the biodistribution and migration of transplanted or transfused stem cells in vivo. The recent paper by Arbab et al represents a significant step forward in bringing the prospect of magnetic resonance imaging cell tracking into the clinic. They describe the use of protamine...
sulfate as a small cationic transfection agent (TA) to shuttle Food and Drug Administration (FDA)-approved ferumoxides (Fe) magnetic nanoparticles (Feridex IV, in an off-label application; Berlex Laboratories, Wayne, NJ) into cells. As protamine sulfate has been approved by the FDA as well (to reverse heparin anticoagulation), and thus also exists as a pharmaceutical preparation, clinical implementation will likely be facilitated.

In this work, the authors have studied the effect that the TA-Fe labeling may have on the differentiation of human mesenchymal stem cells (hMSCs) into adipocytes, osteoblasts, and chondrocytes. Although the viability and proliferation remain unaltered, they reported no adverse effect on cellular differentiation. However, in Figure 2C-D, they failed to demonstrate chondrogenic differentiation, as evidenced by lack of positive (deep red) Safranin-O staining, sections were reacted against monoclonal antibody C4F6; the brown positive signal was composed of precipitated di-amino-benzidine (DAB). Prussian Blue staining revealed Fe-containing cells throughout the pellet (B), but fail to demonstrate chondrogenic differentiation (D-F). To further prove that Fe labeling inhibits chondrogenic differentiation, experiments were performed with 50:50 mixtures of TA-Fe–labeled and unlabeled MSCs (G-I). Only unlabeled, non–Fe-labeled cells (Prussian Blue–negative region in panel H) demonstrate chondrogenesis. Thus, the inhibition of chondrogenesis is mediated by Fe, and not by the TA, as MSCs labeled with only TA (J-L) differentiate normally. All experiments were performed with the same donor cells and passage number. Bar in panel A represents 400 μm. Thin sections of pellets were cleared though xylene and ethanol, then rehydrated. For collagen II indirect immunostaining, sections were reacted against monoclonal antibody C4F6; the brown positive signal was composed of precipitated di-amino-benzidine (DAB). Prussian Blue and safranin O stains were performed as previously described. Sections stained for collagen II and safranin O were also briefly exposed to hematoxilin, to color nucleus blue.

We have extensive experience with MSCs and the process of chondrogenic differentiation,\textsuperscript{3,4} as well as magnetic labeling of MSCs using TA-Fe.\textsuperscript{5-7} Using poly-L-lysine (PLL) as a TA, we have labeled hMSCs with Fe at half the dose that was used by Arbab et al\textsuperscript{2} (25 μg Fe/mL). This results in an intracellular iron incorporation of 13 to 16 pg Fe/cell,\textsuperscript{6,8} in the same range as obtained by Arbab et al (11 pg Fe/cell).\textsuperscript{2} Although we found indeed no adverse effect on cell viability, proliferation, adipogenesis, or osteogenesis, we were surprised to observe inhibition of chondrogenesis in the hMSC donors who were studied (Figure 1). The inhibition appeared dose dependent, as halving the Fe concentration induced limited chondrogenesis (data not shown). The inhibition effect was found to be mediated by the Fe itself, and not the TA (ie, PLL) that was used (Figure 1J-L), indicating that the use of other TAs, including protamine sulfate, may not circumvent the observed chondrogenesis inhibition.

We believe our inhibition results to be noteworthy. They demonstrate, for the first time, that cellular differentiation into a specific cell type can be affected by Fe labeling while the viability and proliferation remain normal. Future studies will need to implement careful ferumoxide titration and cell differentiation studies, in particular now that clinical trials using magnetically labeled cells are being considered.\textsuperscript{9}

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References

We read with interest the results of Bulte and colleagues indicating ferumoxide (Fe)–poly-L-lysine (PLL) complexes inhibited chondrogenic differentiation of mesenchymal stem cells (MSCs) following labeling. They have also raised concerns that the staining characteristics of the cell pellets presented in Figure 2C-E of our report failed to demonstrate chondrogenic differentiation of MSCs labeled with ferumoxide–protamine sulfate (Fe-Pro) complexes.\(^1\) Their comments and data provide us with the opportunity to elaborate on our findings and present additional data that clearly demonstrate chondrogenesis from Fe-Pro–labeled MSCs.

We used MSCs and media from the same supplier as the Bulte lab (Cambrex, Baltimore, MD) and procedures designed to result in differentiation of MSCs along different lineages. Chondrogenic differentiation and expression of collagen II depend on incubation time in chemically modified differentiating media and concentrations of factors for differentiation.\(^2\) Our original results indicate a similar pattern (ie, morphology and size) and extent of staining for proteoglycans and collagen II in chondrogenic-differentiated cells from both Fe-Pro–labeled and unlabeled MSCs.\(^3\)

To further demonstrate that Fe-Pro–labeled MSCs undergo chondrogenic differentiation, labeled and unlabeled cell pellets cultured in appropriate differentiation media for 20 days were stained for proteoglycans with Alcian blue 8GX at pH 2.5 (Sigma, St Louis, MO) and for expression of collagen X. Similar to Safranin-O stain, Alcian blue also stains glycosaminoglycans. Figure 1 demonstrates a similar distribution of Alcian blue stain in the Fe-Pro–labeled and unlabeled cells, along with similar cellular morphology and expression of collagen X in both cell pellets,\(^3\) indicating that Fe-Pro–labeled MSCs are indeed capable of chondrocytic differentiation. Prussian Blue staining of the section clearly shows iron-labeled cells (Figure 1D-E).

The differences between the Fe-PLL and Fe-Pro methods may in fact be responsible for the differences in observations between our lab and the Bulte lab. Our group developed and has extensively characterized the approach for magnetic cell labeling combining ferumoxides with transfection agents.\(^4\) We have shown that labeling stem cells and other cells with Fe-Pro is more efficient and results in a cleaner preparation compared with Fe-PLL, especially if heparin is included in the initial washes.\(^5\) We have been able to duplicate Bulte et al’s observation by incompletely washing Fe-Pro–labeled MSCs before putting them into differentiation media and observed that extracellular iron complexes could be preventing chondrocytic differentiation (Figure 1D). The dose-dependent effect of Fe-PLL on chondrogenesis from MSCs observed by Bulte’s group may be attributable to increasing extracellular complexes with increasing doses of the Fe-PLL label.

In conclusion, our results indicate that chondrocytic differentiation is not inhibited when MSCs are labeled using the Fe-Pro complex. We believe that the observations of Bulte and colleagues using Fe-PLL are interesting but most likely due to differences in labeling techniques and not simply to the presence of ferumoxides in cells. Our results underscore the superiority of the Fe-Pro approach for magnetic cell labeling.

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Response:

Feride–protamine sulfate labeling does not alter differentiation of mesenchymal stem cells

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To the editor:

Important publication missing key information

Gris and colleagues report on a randomized controlled trial comparing 100 mg aspirin to 40 mg enoxaparin started at 8 weeks gestational age in women with prior fetal loss later than 10 weeks and thrombophilia (factor V Leiden, prothrombin gene variant, protein S deficiency). The authors report an astounding 57% absolute risk reduction with enoxaparin compared with aspirin. This absolute risk reduction translates into a number needed to treat of 1.7; that is, 1.7 women with thrombophilia and a loss later than 10 weeks need to be treated with enoxaparin throughout pregnancy to prevent one fetal loss between 8 weeks and term compared with aspirin. Late fetal loss is a very common problem occurring in 2.3% of pregnancies. This dramatic finding may have a large impact on the care of women with prior late fetal loss. The case for thrombophilia screening and prophylaxis of these women is strengthened.

However, the authors, the editors, and the reviewers of this article failed to ensure that essential information was included in the article that would permit the reader to determine the strength of the internal and external validity of the study. Table 1 fails to display baseline characteristics according to treatment groups (aspirin versus enoxaparin) as is suggested in the Consolidated Standards of Reporting Trials (CONSORT) guidelines. By failing to display this information, the reader is unable to determine whether known and important prognostic factors (eg, maternal age) were equally balanced between the treatment groups. The absence of a figure to display flow of trial participants as suggested by the CONSORT guidelines does not permit the reader to determine generalizability of the findings and assess important internal validity issues. When were these patients recruited (ie, over what time frame)? Where and how were patients screened for enrollment? How many screened patients were excluded and for what reasons? How many consenting patients received the intended treatment (ie, treatment as allocated by randomization)? The method of allocation concealment was not described. How many complied completely with the intended treatment? How many were lost to follow-up? How many were analyzed for the primary outcome? Was the analysis done by intention to treat? Were there any interim analyses?

By not reporting these important study characteristics we cannot be reassured that bias is not driving these dramatic results, nor can we be sure that these results apply to our patients.

The authors should be congratulated on completing this study. Performing drug intervention trials in pregnancy is extremely challenging, fraught with medicolegal challenges, regulatory hurdles, and an absence of interest from the pharmaceutical industry. However, prior to adoption of this study’s findings, this important information needs to be revealed.

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References


Response:

Thromboprophylaxis for first fetal loss

We would like to thank Dr Rodger for his comments on our study, a nested trial in the case-control study “NOHA big first,” the results of which are not yet published.

The aim of the Nîmes Obstetricians and Haematologists (NOHA) big first, which began on January 1, 1999, was to clarify the risk factors for a first pregnancy loss.

Obstetricians and gynecologists from the southern French region of Languedoc sent us patients who experienced a pregnancy loss during their first or second pregnancy. Our multidisciplinary staff selected these cases of unexplained pregnancy loss, and 97.8% of them were investigated at the outpatient department of hematology. Patients with an obvious medical etiologic factor (1.15%) or thromboembolic antecedents (3.4%) were excluded, and 98.8% of the residual patients agreed to join the case-control study (n = 3496). There were 186 patients who were heterozygous for the factor V Leiden mutation (FVL), 127 for the prothrombin 20210G>A mutation (F2M), and 31 with a protein S insufficiency (PS). Of them, 69% (who were negative for classical antiphospholipid antibodies and hyperhomocysteinemia) were potentially eligible for our therapeutic trial (FVL: n = 127; F2M: n = 85; PS: n = 25; total: n = 237).

The therapeutic trial began January 1, 2001, and ran until January 1, 2003. Participation in this trial was proposed to the aforesaid patients with pregnancy loss from the 10th week (n = 94)
Chondrogenic differentiation of mesenchymal stem cells is inhibited after magnetic labeling with ferumoxides

Jeff W. M. Bulte, Dara L. Kraitchman, Alastair M. Mackay and Mark F. Pittenger