Elevated C-met in Thymic Dendritic Cells of New Zealand Black Mice

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New Zealand Black (NZB) mice are a well-known animal model of human autoimmune disease. Although the mechanism for development of autoimmunity is unclear, NZB mice are well known for severe thymic microarchitecture abnormalities. It is thought that thymic dendritic cells (DC) may play a role in thymic education and contribute to the autoimmune process. To address this issue and, in particular, that qualitative and/or quantitative differences exist in thymic DC, we took advantage of a novel restriction analysis system that allow definition of differences in the expression of tyrosine kinases using highly enriched populations of thymic DC from NZB compared to BALB/c and C57BL/6 mice. The method chosen, restriction analysis of gene expression, allowed the determination of protein tyrosine kinase transcription profiles. We report herein that NZB mice have a significant upregulation of C-met compared to the control strains. The abnormality of the C-met transcription was confined to thymic DC. We believe that its abnormal expression reflects the resistance of thymic cells to apoptosis, which will ultimately lead to defects and/or abnormal signaling by the interaction of thymic DC and thymocytes. Further studies involving such interactions are under way.

Keywords: C-met; Lupus; Thymic dendritic cells; New Zealand black mice

INTRODUCTION

The New Zealand Black (NZB) mouse develops an autoimmune disease characterized by high serum IgM levels and autoantibody production that progresses with age (Borchers et al., 2000). Recently our laboratories have shown abnormalities in thymic microarchitecture involving epithelial cell network (Watanabe et al., 1993; Takeoka et al., 1999) and thymic B cells (Taguchi et al., 2001a,b) in the NZB mouse. The mechanisms for these changes and the relationship to autoimmunity are unknown.

Thymic dendritic cells (DC) originate from the bone marrow and reside within the thymic medulla or at the cortico-medullary junction (Ardavin, 1997) and play a pivotal role in thymocyte education, particularly in the context of negative selection (Anderson et al., 1996; Brocker et al., 1997; Ferrero et al., 1997; Brocker, 1999). It has been previously shown that this process of negative/positive selection is, to a large extent, mediated by signals generated intracellularly following lymphoid cell surface interaction with corresponding ligands expressed by cells involved in the selection process, which includes thymic DC. We hypothesized that qualitative and/or quantitative differences may exist in the ability of thymic DC to mediate T cell selection in NZB mice which would contribute to the autoimmune disease in such mice. The recent description of a novel restriction analysis of gene expression (RAGE) (Robinson et al., 1996; Lin et al., 2000), which can be performed on small numbers of cells, prompted us to use this technique for defining differences (if any) in the expression of tyrosine kinases by highly enriched populations of thymic DC from NZB as compared to BALB/c and C57BL/6 mice. The tyrosine kinases were chosen based on an extensive body of data that indicates the importance of such kinases as regulators of intracellular signal-transduction mediating molecules and the mediation of intracellular communication (Blume-Jensen and Hunter, 2001).

We report herein a marked selective thymic DC up-regulation of C-met tyrosine kinase mRNA in NZB mice. This is the first report of an abnormal transcription of a
gene that may play an important role in apoptosis and cell–cell adhesion within the NZB thymus. These abnormalities may result in the escape of autoimmune T cells, with development of the resulting disease phenotype.

MATERIALS AND METHODS

Mice

BALB/cJ, C57BL/6J, and NZB/BINJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred at the Animal Resource Services Facility at the University of California, Davis. Four to five weeks-old mice were used for these experiments.

Antibodies and Reagents

FITC-conjugated rat anti-mouse MHC class II (M5/114.15.2) antibody was purchased from Miltenyi Biotec (Auburn, CA). FITC-conjugated rat anti-mouse CD8α (53-6.7), PE-conjugated hamster anti-mouse CD11c (HL3), FITC and a series of biotin-conjugated rat anti-mouse CD45R/B220 (GL1), rat anti-mouse CD80 (B7.1), biotin conjugated anti-mouse CD86 (B7.2), or biotin conjugated anti-mouse CD40. Biotinylated antibodies were visualized using tricolor streptavidin. Prior to staining, the FcγRI/III receptor on cells were blocked by preincubation of the samples with an anti-mouse CD16/32 antibody to reduce non-specific staining. Cells were incubated with primary antibodies in staining buffer (0.2% BSA and 5 mM EDTA) for 30 min at 4°C, washed in washing buffer (0.2% BSA and 5 mM EDTA in PBS), and incubated with tricolor streptavidin in staining buffer for an additional 30 min at 4°C. After the last wash, the cells were resuspended in washing buffer and viable cells from each sample were analyzed using flow cytometry (FACScan, BD) and the data analyzed using Cell Quest software (BD). To isolate thymic DC, thymic CD11c⁺B220⁻ MHC classII⁺ cells from DC-enriched preparation were collected by 10-parameter MoFlo cell sorter (Cytomation, Fort Collins, CO) equipped with Summit software. The frequency of thymic DC was always >95% in the preparations utilized for the studies reported herein.

Splenic DC Isolation

Splenic DC enrichment was performed essentially as described above for thymic DC enrichment. Splenic DC were treated with rat anti-mouse B220 mAb followed by incubation with sheep anti-rat IgG-conjugated magnetic beads (Dynal, Oslo, Norway). Passage through a magnetic field was used to deplete the B220⁺ cells. Such enriched splenic B220⁻ cells were subsequently incubated with CD11c microbeads (Miltenyi Biotec) and the CD11c⁺ cells isolated and subjected to further enrichment using a cell sorter. The frequency of splenic DC was always >90% for the assays utilized in the studies reported herein.

RNA Extraction and cDNAs Synthesis

Total RNA was extracted from splenic and thymic DC from BALB/c, C57BL/6, and NZB mice utilizing the RNeasy kit (QIAGEN, Valencia, CA). cDNAs were synthesized using Superscript™ II (Life Technologies, Grand Island, NY) and oligo dT(12–18) primer (Life Technologies).

RAGE Analysis for Tyrosine Kinases

The RAGE assay was carried out using a modification of the method previously described (Lee et al., 1999). Essentially, reverse transcription-polymerase chain reaction (RT-PCR) was performed with degenerate primers derived from conserved motifs in the activation loop of the catalytic domains of various tyrosine kinases as follows; primer 1 (sense primer), 5'-AAR RTT DCN GAY TTY GG encoding the amino acid sequence...
K[VI][S/C/G]DFG; primer 2 (antisense primer), 5'-RHA 
IGM CCA IAC RTC encoding the amino acid sequence 
DVW[S/A][F/Y]. The mixed bases were defined as 
follows: N = A + C + T + G, D = A + T + G, 
H = A + T + C, R = A + G, Y = C + T, M = A + C, 
and I = deoxyinosine. Primer 1 was labeled with [γ-32P] 
ATP (NEN Life Science Products, Boston, MA) and T4 
polyadenylate kinase (Life Technologies) and PCR was 
performed by using 32P labeled primers1, unlabeled 
primer2, and AmpliTaq Gold DNA polymerase (Perkin– 
Elmer, Boston, MA). The PCR products were electro-
phoresed in a 2.4% agarose gel (3:1 ratio of Nusieve 
and regular agarose; BMA, Rockland, ME). The 
153–177 bp bands were excised from the gel and DNA 
eluted using the QIAEXII gel extraction kit (QIAGEN). 
Samples were standardized by adjustments based on 
radioactivity as determined by radioactive scintillation. 
Equal amounts of radioactive DNA of each sample were 
digested with Accl, AluI, AvaII, BsrI, Ddel, HaelIII, HinfI, 
Hpall, MnlI, RsaI, or TaqI restriction enzyme (New 
England Biolabs, Beverly, MA) resolved on a 7% DNA 
sequencing gel, and exposed overnight at –70°C on a 
fluorescent screen for quantitative analysis on a Phos-
phoimager® (Molecular Dynamics, Sunnyvale, CA).

RT-PCR

Serial dilutions of cDNA were PCR-amplified for c-met, 
Bcl-Xi, or β-actin using AmpliTaq Gold DNA polymerase 
in the presence of either c-met primers (5'-CCA GCA 
GCT TCA GTT ACC GG-3', 5'-GGC ATG CTG ACA 
TGC CAC TG-3') Bcl-Xi primer (5'-TGA TTC CCA TGC 
CAG CAG TGA-3', 5'-AAC CAC ACC AGC CAC AGT 
CAT-3'), or β-actin primer (5'-CTT AAG GCC AAC 
CGT GAAAAG-3', 5'-TCT TCA TGG TGC TAG GAG 
CCA-3'). Amplified products (c-met 182 bp, Bcl-Xi, 
434 bp, β-Actin 646 bp) were electrophoresed on a 1.5% 
agarose gel and visualized with ethidium bromide. In these 
studies the c-met primers were designed using Primer 
Express 1.0 software (Applied Biosystems, Foster, CA). 
In addition, the resulting PCR product of 182 bp which 
corresponds to the expected c-met mRNA-derived signals, 
was confirmed to be the c-met gene by direct sequencing 
of the PCR product.

Real-Time PCR

Analysis was performed using the GeneAmp 5700 
Sequence Detection system (Perkin–Elmer) utilizing 
SYBR™ Green (Molecular Probes, Eugene, OR) as descri-
bred (Taguchi et al., 2001a). Primers for c-met were the 
same as used in the RT-PCR experiment. β-actin primers 
(5'-ACT ATT GGC AAG GAG CGG TT-3', 5'-CAG GAT 
TCC ATA CCC AAG AAG GA-3') were designed using 
Primer Express 1.0 software (Perkin–Elmer). Standard 
curves were generated using diluted cDNA of thymic 
CD11c+ cells for c-met or β-actin. To measure relative 
intensity between normal and NZB mice, the ratio of 
c-met mRNA level was calculated as c-met intensity 
divided by β-actin intensity. All reaction wells were run in 
duplicate and included control wells without cDNA.

RESULTS

Thymic Dendritic Cell Frequency in NZB Mice

Thymii from groups of NZB and for purposes of control, 
BALB/c and C57BL/6 mice were individually analyzed 
for the frequency of thymic DC (mean ± SEM). The 
NZB thymic DC frequency (mean ± SEM) (4.07 ± 0.45 × 10^4/10^8 total thymocytes) was similar to 
that of control mice (BALB/c; 3.39 ± 0.36 × 10^4/10^8, 
C57BL/6; 4.08 ± 0.35 × 10^4/10^8 total thymocytes). The 
level of expression of cell surface markers in NZB thymic DC 
was not significantly different from control thymic DC 
(MHC II^high, CD8α^+, CD40^+, B7.1^+, B7.2^+, data not shown).

Determination of Protein Tyrosine Kinase 
Transcription Profile in NZB Thymic DC

We next carried out studies to document the profile of 
tyrosine kinase utilizing the RAGE assay expressed by 
highly enriched populations of one month old BALB/c 
and NZB thymic DC (CD11c^+CD220^- cells). Amplified 
PCR products containing a series of tyrosine kinases were 
digested with the restriction enzymes and the intensity 
of bands compared. Repeated analysis of visualized bands 
led to the identification of three bands which had higher 
signal intensity in NZB thymic DC. Based on the associa-
tion between the restriction enzyme used and the length 
of band obtained, the BsrI digested 92 bp band corresponded 
to the Frk, C-met, and/or, Ron tyrosine kinases (Fig. 1a). 
The HinfI digest led to a 115 bp band which was identified 
to be C-met tyrosine kinase (Fig. 1b). The Rsal digested 
44 bp band corresponded to Ddr2, C-met, and/or Trk-B 
tyrosine kinases (Fig. 1c). Thus, C-met kinase appeared to 
be the common tyrosine kinase identified by these restric-
tion enzyme digests.

Identification of PTK mRNA that are Differentially 
Expressed by NZB Thymic DC

In efforts to confirm the above difference in c-met at the 
mRNA level, RT-PCR analysis was performed using 
c-met specific primers. Figure 2a shows that the c-met 
mRNA level in NZB thymic DC was higher than control 
strains. To improve sensitivity and quantitation, real-time 
PCR was performed which included the β-actin primers. 
As seen in Fig. 2b, NZB thymic DC contained a higher 
level of c-met mRNA. Thus, the RT-PCR and real time 
PCR data concur with the RAGE data and clearly 
demonstrates that c-met mRNA levels in NZB thymic DC 
is higher than control mice.
c-met mRNA by Thymocytes and Splenic DC

To ascertain the lineage specificity of the increased expression of c-met mRNA, a comparative analysis of c-met mRNA levels was carried out on total thymocytes and splenic DC by real time-PCR. Interestingly, thymocytes from control mice and NZB mice had a much lower c-met mRNA level (Fig. 3) than corresponding thymic DC. In addition, it was of interest to note that the c-met mRNA level in NZB splenic DC was similar to control thymic and splenic DC. These data suggest that the abnormal expression on c-met mRNA is restricted to the population of NZB thymic DC and not a global dysregulation of c-met expression.

DISCUSSION

In systemic lupus erythematosus (SLE) patients, the Fyn tyrosine kinase activity of T cells from peripheral blood has been shown to be elevated when compared to healthy donors (Blasini et al., 1998). Using a variety of tyrosine kinase family of mutated or knockout mice, it was reported that such tyrosine kinases are involved in the development of SLE like disease (Morino et al., 1999; Lu...
and Lemke, 2001; Yu et al., 2001). These data provided the basis for our rationale to screen for all amplified tyrosine kinases in efforts to identify the spectrum of tyrosine kinases that may potentially dysregulated in autoimmune prone NZB mice as compared with normal BALB/c and C57BL/6 mice.

C-met kinase is a receptor for the hepatocyte growth factor (HGF) and its gene has been localized to mouse chromosome 6 (Chan et al., 1988). C-met is expressed within a number of cell lineages, including epithelial cells (Di Renzo et al., 1991), endothelial cells (Busolino et al., 1992), myoblasts (Anastasi et al., 1997), and hematopoietic cells (Galimi et al., 1994; Nishino et al., 1995). Interaction of HGF with c-met activates multiple intracellular signaling pathways involved in muscle and liver formation (Schmidt et al., 1995; Maina et al., 1996), cell proliferation, morphogenesis, and motility. In contrast to thymic DC, c-met mRNA levels in NZB splenic DC was similar to control splenic DC, suggesting that either the elevation of c-met mRNA levels in NZB thymic DC might be independent of a defect of the entire NZB DC population, or thymic DC may comprise a sublineage of DC with unique homing properties to the thymus.

It is unknown why the c-met transcription abnormality is confined to the thymic DC population in NZB mice. Among the potential reasons entertained is our previously documented thymic architectural abnormalities in NZB mice (Watanabe et al., 1993; Takeoka et al., 1999). HGF/c-met interaction exerts an upregulation of anti-apoptotic related genes (Aebersold et al., 2001) and downregulation of E-cadherin (Miura et al., 2001) and adhesion molecules, mediated by β-catenin (Behrens et al., 1993; Danilkovitch-Miagkova et al., 2001). From our data, the anti-apoptotic factor, Bcl-XL mRNA in NZB thymic DC was significantly higher than control strains of mice. C-met upregulation in thymic DC may contribute to resistance against apoptosis. It is reasoned that the elevation of c-met in NZB thymic DC causes a decrease in the levels of E-cadherin, which leads to defects and/or abnormal signaling by the interaction between thymic DC and thymocytes. It is of interest to note that significant levels of c-met have been documented to be expressed by fetal thymus, liver and hematopoietic cells (Selden et al., 1990; Hu et al., 1993; Galimi et al., 1994; Tamura et al., 1998) but to a lower level by adult thymic and splenic DC in normal mice. Preliminary studies of highly enriched populations of adult thymic CD3⁻CD4⁻CD8⁻ cells (triple negative thymocytes also known as progenitors for T cells and DC) also expressed significantly higher levels of c-met than adult thymocytes (data not shown). These data support the concept that c-met expression may be a marker of immature cells. Thus, such immature NZB thymic DC may not optimally function in the deletion of autoreactive T cells.
It would be important to confirm the relative levels of RNA with levels of c-met protein expression. However, this could not be examined on thymic DC because it was difficult to collect enough cells to carry out such studies. In addition, in efforts to clarify the mechanism of c-met elevation by NZB thymic DC, a study of HGF and adhesion molecules should be performed. Finally, since the data described herein imply the potential of a growth arrest of thymic DC progenitor cells which may be a source of abnormalities in the NZB mouse, they will be closely examined in the context of c-met in future experiments.

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