Increased Expression of Monocyte CD11b (Mac-1) in Overweight Recent-Onset Type 1 Diabetic Children

Vincenza Cifarelli¹, Ingrid M. Libman², Angela DeLuca³, Dorothy Becker², Massimo Trucco¹ and Patrizia Luppi¹

¹ Division of Immunogenetics, Department of Pediatrics, Children’s Hospital of Pittsburgh, University of Pittsburgh, School of Medicine, Pittsburgh, PA 15213, USA. ² Division of Endocrinology, Department of Pediatrics, Children’s Hospital of Pittsburgh, University of Pittsburgh, School of Medicine, Pittsburgh, PA 15213, USA. ³ Department of Experimental Medicine, Section of Human Anatomy, University of Palermo, School of Medicine, Italy. Address correspondence to: Patrizia Luppi, e-mail: luppip+@pitt.edu

Abstract

AIM: Compelling evidence implicates inflammation in the pathogenesis of type 1 diabetes mellitus (T1DM) and associated vascular complications. Obesity is also characterized by low-grade systemic inflammation. In this study, we characterized the inflammatory response in diabetes by analyzing the expression of a panel of activation markers on the surface of peripheral blood monocytes in recently-diagnosed T1DM patients. The potential effects of glycemic control and of body mass index (BMI) on monocyte phenotype was also investigated. METHODS: Using flow cytometry, we analyzed the expression of CD11b, CD49d, CD54, CD62L, and CD64 antigens on monocytes in a cohort of 51 T1DM patients (≤ 2 months after diagnosis). RESULTS: We found that circulating monocytes from T1DM patients tested at the clinical onset of the disease (i.e. within 1 week of diagnosis) had higher CD11b expression compared to patients analyzed 2 months after diagnosis (p = 0.02). The highest CD11b levels were detected in patients with HbA1c > 8% (p = 0.04 vs. patients with HbA1c < 8%). In T1DM children analyzed 2 months after diagnosis, we found that those who were overweight (BMI ≥ 85th percentile) had higher levels of monocyte activation than those who were not overweight (BMI ≤ 85th percentile) (p = 0.03). CD11b and HbA1c were significantly correlated (correlation coefficient 0.329, p = 0.02). CONCLUSIONS: Circulating immune cells from T1DM patients display many aspects of a proinflammatory state, as indicated by primed or activated monocytes. Obesity is an important factor in monocyte activation during diabetes.

Keywords: monocytes · adhesion molecules · type 1 diabetes · obesity · inflammation

Introduction

Compelling evidence demonstrates that components of the innate immune system, including natural killer cells (NK) and monocytes are involved in the autoimmune response characteristic of T1DM both in humans and in the non-obese diabetic (NOD) mouse [1-4]. The primary role of monocytes in T1DM has been demonstrated by showing that these cells are the first to accumulate in the pancreatic islets of prediabetic BB rats [5]. Subsequent T and B lymphocyte infiltration is dependent upon prior monocyte invasion of the islets [5]. These data suggest a role for monocytes in the early stages of T1DM pathogenesis [6].

Monocytes are pivotal cells in inflammatory responses as they serve as the principal reservoir of pro-inflammatory cytokines and are the first cells to be engaged in nonspecific immune responses, such as those triggered by environmental factors. Recent studies re-
In these patients, monocytes also released higher levels of markers of inflammation and oxidative stress in adult T1DM patients well after the onset of diabetes [7, 8]. Reported evidence of increased monocytic activity, bio-markers of inflammation and oxidative stress in adult diabetic subjects, because it is not possible to follow directly the different stages of the islet inflammatory process. Changes that affect both the vasculature and circulating monocytes in the early stages of T1DM may play a crucial role in promoting leukocyte adherence to the endothelium and ongoing infiltration of the islets. Another factor that could affect monocyte activation during diabetes is not fully understood. This is of particular importance in human subjects, because it is not possible to follow directly the different stages of the islet inflammatory process. Changes that affect both the vasculature and circulating monocytes in T1DM patients is changes in body weight, as obesity has been associated with the presence of leukocyte abnormalities and inflammation [9, 17, 18]. In this study, we therefore investigated the potential role of glycemic control and body mass index (BMI) on monocyte expression of a panel of adhesion molecules in recently-diagnosed T1DM children (≤ 2 months after diagnosis).

### Materials and Methods

#### Subjects

Fifty-one T1DM patients with recently diagnosed type 1 diabetes (T1DM) (i.e. within two months of the first insulin injection) (mean age 8.7 ± 4.4 SD, 22 F: 29 M) were randomly selected from those treated in the Diabetes Center at the Children’s Hospital of Pittsburgh (CHP) between the years 2003-2004. Of those patients, thirty-eight were tested within one week of clinical diagnosis (defined as “new-onset”), while thirteen were tested at 2 months from clinical diagnosis. In the new-onset group, twelve patients had diabetic ketoacidosis (DKA), as defined by the presence of blood bicarbonate levels < 18meq/l. The demographic and clinical characteristics of the population studied are shown in Table 1. Of the fifty-one diabetic patients, twenty-three tested positive for at least one autoanti-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treated at onset</th>
<th>Tested ≥ 2 months of onset</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (n)</td>
<td>38</td>
<td>13</td>
<td>51</td>
</tr>
<tr>
<td>Male/female (%)</td>
<td>50/50</td>
<td>77/23*</td>
<td>57/43</td>
</tr>
<tr>
<td>Caucasian/biracial (%)</td>
<td>97/3</td>
<td>85/15</td>
<td>94/6</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>7.9 ± 4.1</td>
<td>10.9 ± 4.5*</td>
<td>8.7 ± 4.4</td>
</tr>
<tr>
<td>BMI percentile**</td>
<td>52.3 ± 34.7</td>
<td>70.9 ± 33.2</td>
<td>57.8 ± 34.9</td>
</tr>
<tr>
<td>Diabetic ketoacidosis*** (%)</td>
<td>38</td>
<td>0*</td>
<td>27.6</td>
</tr>
<tr>
<td>HbA1c*** (%)</td>
<td>11.3 ± 2.0</td>
<td>7.4 ± 0.9*</td>
<td>10.2 ± 2.5</td>
</tr>
</tbody>
</table>

Legend: Data are mean ± SD, numbers or percentages. BMI: body mass index. *p < 0.05 for comparisons between the two groups. ** Data available in 44 children (31 in group 1 and 13 in group 2). *** Data available in 47 children (34 in group 1 and 13 in group 2).
body: GAD65, insulinoma-associated protein 2 (IA-2) and/or insulin autoantibodies (IAA) [19]. Each of the patients gave his informed consent to participate in the study, which was approved by the Children’s Hospital Institutional Review Board.

Sample processing

Peripheral venous blood from T1DM patients was collected in sodium heparin tubes, kept at room temperature and processed within three hours for flow cytometry staining. Monocyte isolation was performed on Ficoll-isolated peripheral blood mononuclear cells (PBMC) and used for adhesion experiments as described below.

Flow cytometry

Sample labeling was performed as follows. Fifty microliters of whole blood from each subject were incubated with 20 µl of the appropriate dilutions of anti-human monoclonal antibodies (mAbs) and isotype matching mAbs for 15 minutes, in the dark, at room temperature. After lysing the erythrocytes with 2 ml 1x PharM Lyse™ (BD Pharmingen, San Diego, CA), samples were stored in 1% paraformaldehyde at 4°C until measured by flow cytometry between 5 minutes and 4 hours later [20].

The following anti-human mAbs were used: phycoerythrin (PE)-conjugated mAb to -CD62L (BD Pharmingen), -CD54 (BD Biosciences, San Diego, CA), and -CD49d (BD Pharmigen), Cy-chrome-conjugated mAb to -CD64, and -CD11b/Mac-1 (BD Pharmigen) and allophycocyanin-(APC)-conjugated mAb to -CD14 (BD Biosciences). Each mAb was used at saturating concentrations optimized by initial titrations for flow cytometry staining. The negative control panels were comprised of mixtures of isotype controls (BD Pharmingen, BD Biosciences) diluted to an identical immunoglobulin concentration.

Flow cytometry and data analysis were undertaken as follows. Blood cells were analyzed using a Becton Dickinson (San Jose, CA) FACS Calibur machine calibrated with CaliBRITE™ beads. At least 30,000 events were collected for each sample and the data were saved for later analysis on CELLQuest software (Becton Dickinson). Analysis of the expression of CD11b on the surface of circulating monocytes was performed by setting specific gates for the monocyte population based on dot plot graphs of side scatter (SSC) versus the positive cells for the antibody specific to the monocyte surface marker CD14 (FL4).

Monocytes from new-onset T1DM patients have an activated phenotype

Analysis of the mean fluorescence intensity (MFI) of the monocyte marker CD11b in children with T1DM, showed a higher expression in those who were tested at the clinical onset of diabetes (n = 38, within 1 week of diagnosis) (878 ± 83 SEM, range 210-2403, median 698) compared to those tested at 2 months after diagnosis (n = 13) (595 ± 132 SEM, range 180-1663, median 442), (p = 0.02) (Figure 2 and Figure 3). Because diabetes ketoacidosis (DKA) has been described as an inflammatory condition characterized by
elevated levels of C-reactive protein, within our cohort of newly-onset T1DM patients, we found that mono-
cyte expression of CD11b in the children with DKA
tended to be higher (1007 ± 188 SEM) compared to
those without DKA (797 ± 82 SEM). However, this
was not statistically significant (p = 0.67). When we
compared those without DKA at onset (n = 21) with
those without DKA at 2 months (n = 13), differences
in the monocyte expression of CD11b was statistically
significant between the two groups (797 ± 82 versus
595 ± 132 respectively, p = 0.03).

As expected, glycemic control measured by HbA1c
was significantly higher in the onset group compared
to the group tested at 2 months (11.3% ± 2 versus
7.4% ± 0.9, p = 0.0001). To test whether patients with
higher HbA1c had also higher monocyte activation, we
compared the expression of monocyte CD11b in T1DM
patients according to HbA1c levels. We found that
T1DM patients with higher HbA1c levels (HbA1c
> 8%) (n = 35) had significantly higher expression of
CD11b (896 ± 75 SEM) as compared to those with
lower HbA1c levels (HbA1c ≤ 8%) (563 ± 103 SEM)
(n = 12) (p = 0.04). In addition, CD11b and HbA1c
were significantly correlated (correlation coefficient
0.329, p = 0.02).

Analysis of the expression of the surface antigens
CD49d, CD54, CD62L and CD64 on circulating
monocytes in the different subgroups of patients did
not reveal any significant differences. A representative
example of the expression of the stated markers in
T1DM patients at the onset of the disease and 2
months after diagnosis is shown in Figure 3.

Overweight children with T1DM have high monocyte expression of CD11b

There is evidence that obesity is associated with a
systemic inflammatory process involving both leuko-
cytes and body fat [17, 18]. We therefore analyzed
changes in monocyte CD11b expression in T1DM
according to BMI. We found that among T1DM
children, who were tested 2 months after diagnosis (n = 13), expression of CD11b was significantly higher in
overweight children (BMI ≥ 85th percentile) (n = 7)
(844 ± 201 SEM, median 531, range 332-1663) as
compared to children who were not overweight (BMI
≤ 85th percentile) (n = 6) (305 ± 61 SEM, median 233,
range 180-540) (p = 0.03) (Figure 4A). These two
groups were no different in terms of their glycemic
control as measured by HbA1c (7.1% ± 0.3 in the
overweight group versus 7.6% ± 4.5 in the non-
overweight group, p = 0.33) and none of them were in
DKA at the time of the evaluation.

The same analysis performed in patients at the on-
set of the disease (n = 31) did not show significant dif-
fferences in monocyte CD11b between overweight
(BMI ≥ 85th percentile) (n = 9) (864 ± 97 SEM, me-
dian 713, range 291-1989) and lean (BMI ≤ 85th
percentile) (784 ± 153 SEM, median 543, range 210-1574)
(n = 22) patients p = 0.66 (Figure 4B). These two
groups were no different in terms of their control as
measured by HbA1c (10.4% ± 0.5 in the overweight
group versus 11.6% ± 0.4 in the non-overweight
group, p = 0.33) or the presence of DKA (50 versus
31%, p = 0.65). CD11b and BMI percentile did not
correlate in a statistically significant fashion (correla-
tion coefficient 0.027, p = 0.86).

Conclusions

Little is known about monocyte phenotype and
function in children with recently diagnosed T1DM. In
this study, we analyzed the expression of a panel of
adhesion molecules on circulating monocytes in
T1DM children within 2 months of clinical diagnosis
and evaluated the potential role of glycemic control
and BMI on cell phenotype. We found that, among the
markers analyzed, only the expression of CD11b was
significantly higher in the cohort of new-onset diabetic
patients (i.e. within 1 week of diagnosis) as compared
CD11b is a polypeptide α-chain linked to the β2-integrin family. Resting monocytes constitutively express integrins, which are important signal transducers for virtually all monocyte functions, by mediating cell adhesion, chemotaxis, migration, phagocytosis, and oxidant production. After monocyte activation, new copies of CD11b/CD18 are rapidly translocated to the cell surface from the intracellular granules [21]. Our finding of increased CD11b expression on monocytes at the onset of T1DM suggests the presence of immune activation at such an early stage of the disease. This result also supports an earlier finding of increased monocyte CD11b expression in T1DM patients [14].

Within the cohort of new-onset T1DM patients, children who presented with DKA had the highest levels of monocytes CD11b detected. Although these data did not reach statistical significance, most likely because of small numbers, it supports a previous report showing presence of an inflammatory response in T1DM children with DKA [22]. However, other factors besides DKA appear to be involved in triggering monocyte activation at the onset of diabetes. In fact, significantly higher monocyte CD11b expression was detected in new-onset T1DM children without DKA versus those without DKA at 2 months. These results seem to suggest that the onset of diabetes is per se an inflammatory condition. Further studies on a larger number of patients at the onset of T1DM are required to define better the inflammatory state present in DKA.

We found that diabetic children tested 2 months after diagnosis, who were overweight (BMI ≥ 85th percentile), displayed higher CD11b values when compared to lean diabetic children. This finding supports the association between obesity and inflammation [17, 18]. Recently, it has been shown that obesity is characterized by abnormalities in peripheral leukocyte counts [17], increased circulating levels of the pro-inflammatory cytokine interleukin (IL)-8 [9] and by an accumulation of immune cells, especially macrophages, in the adipose tissue [18]. The fact that we could not detect the same changes in monocyte CD11b between overweight and lean diabetic children within the cohort of patients tested at the onset of the disease is probably due to the confounding effect of the inflammatory response associated with the onset of the disease.

The factor(s) triggering upregulation of CD11b in the monocytes in diabetes and its biological significance are not known. One hypothesis would be that the increased expression of CD11b in monocytes in diabetes is simply a marker of an inflammatory response, which becomes more pronounced in over-
weight children and in children who present with DKA. Alternatively, CD11b upregulation could reflect an increased monocyte activation in response to the poor glycemic control and insulin deficiency which is likely to be present at the onset of diabetes, a hypothesis supported by the fact that HbA1c levels were significantly higher at the onset than at 2 months and by the fact that there was a significant correlation between the two variables. In addition, diabetic children with higher HbA1c (HbA1c > 8%) also had higher CD11b.

A third explanation envisions that intercurrent infections may have precipitated the diagnosis (because of increased insulin requirements) and be responsible for immune activation. At this point, we cannot decide to what extent the inflammatory state is a pathogenic mechanism contributing to the development of T1DM or is the response to a metabolic derangement. In the first case, the inflammatory state should precede overt diabetes and, in this context, the study of pre-diabetic subjects (i.e. autoantibody-positive individuals) should be characterized in respect to the inflammatory state.

Our findings of increased CD11b in the monocytes of T1DM patients at the clinical onset of diabetes

**Figure 4.** Monocyte CD11b expression among T1DM patients is higher in those who are overweight. In A, a column-scatter-plot shows mean fluorescence intensity (MFI) for the molecule CD11b in monocytes according to body mass index (BMI) in thirteen patients tested 2 months after diagnosis (lean n = 6, overweight n = 7). In B, the same analysis is performed in thirty-one T1DM patients at the onset of the disease (lean n = 22, overweight n = 9). Horizontal bars show mean CD11b values. *Represents significant differences in cell surface expression for CD11b between T1DM patients who are overweight (BMI ≥ 85th percentile) and lean (BMI ≤ 85th percentile).

**Figure 5.** Monocytes from T1DM patients have increased adherence to human umbilical vein endothelial cell (HUVEC). Freshly isolated monocytes (1x10⁶ cells/ml) from new-onset T1DM patients (n = 8) and from healthy controls (n = 5) were added to confluent HUVEC in 48 well plates and incubated for 1h at room temperature on a rocking plate. Each experiment was performed in triplicate. After washing away non-adherent cells, adherent monocytes were fixed with 1.25% gluteraldehyde at 4ºC overnight. Next day, monocytes were stained with 0.4% trypan blue and cells were counted. To determine the number of monocyte adherent to HUVEC in each donor, five random 40x fields per well were counted and averaged. 40x representative photographs of monocytes adherent to HUVEC in T1DM patient (A) and non-diabetic control subjects (B) are shown. C gives a column-scatter-dot-plot showing the number of monocytes adherent to HUVEC in 5 random 40x fields per well. Horizontal bars show the mean number of monocytes. The number of monocytes adherent to the wells in T1DM patients was significantly higher than in normal controls (p = 0.04).
Monocyte CD11b Expression in T1DM
The Review of Diabetic Studies
Vol. 4 No. 2 - 2007

would strengthen the idea that these cells have the potential to adhere to the endothelium and exit the circulation at such an early stage of the disease. In fact, current investigations in our laboratory suggest that monocytes isolated from the peripheral blood of recently-diagnosed T1DM patients (who were free from any vascular complications) have increased adherence to human umbilical vein endothelial cells (HUVEC) in vitro (Figure 5). These unpublished findings are preliminary, but they support the hypothesis that monocytes from recent-onset T1DM patients have increased adhesive capacity. Protracted inflammation with increased expression of the same adhesion molecules could be the basis for late diabetes-associated atherosclerotic vascular complications.

In conclusion, the clinical onset of T1DM is associated with changes in activation-related markers in the circulating monocytes as part of a generalized inflammatory response. We have identified the leukocyte integrin CD11b/CD18 as a crucial molecule upregulated in circulating monocytes, especially in overweight children. Enhanced monocyte CD11b would support the notion that monocytes in recently diagnosed T1DM patients have the potential to adhere to the endothelium, most likely in the vasculature of the pancreas, and to accumulate in the islets.

Acknowledgments: We thank the nurses of the Diabetes Clinic and of the Endocrinology Division of the Diabetes Center at the Children’s Hospital of Pittsburgh for making blood samples from Type 1 diabetic patients promptly available to us. We also thank Dr. Sati Mazumdar, Department of Biostatistics, Graduate School of Public Health, for discussion on the statistical analysis. This study was supported by grant DK 024021-24 from the National Institute of Health and NIH SK12 DK063704 and the Cochrane Weber Endowed Fund (I. Libman).

References


