ORIGINAL PAPER

Lymphocyte dysmetabolism: an immunocytochemical comparative approach in IDDM and control subjects

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SUMMARY

We have investigated by immuno-electron microscopy the presence of phosphotyrosine in cells as a whole and in different cell districts (nucleus, cytoplasm, plasma membrane, and mitochondria) in peripheral blood lymphocytes of IDDM (insulin-dependent diabetes mellitns) patients and age-matched controls.

Immuno-gold particle density was highest in mitochondria and decreased in cytoplasm, nucleus and plasma membrane. The time dependence of phosphotyrosine labelling after cell isolation was very strong in all subcellular populations, with a fall in immunogold staining after 30 min.

Staining levels at zero time were similar in controls and IDDM patients; the loss of phosphotyrosine labelling was much stronger in controls, except in the plasma membrane.

Plasma membrane NADH oxidoreductase activity, studied using cytosolic NADH as substrate and assayed with DCIP as acceptor, was significantly reduced in IDDM patients, suggesting a response to a deficient mitochondrial energetic activity.

The fact that NADH oxidoreductase is a growth factor related to tyrosine phosphorylation pathways raises intriguing questions on the cellular derangement occurring in peripheral lymphocytes in IDDM, although the relationships among the immunocytochemical and biochemical changes is still obscure.

INTRODUCTION

It is well documented that several metabolic dysfunctions are present in hyperglycemic conditions; the mechanisms underlying such metabolic complications of hyperglycemia are not, however, completely clear. Experimental diabetes seems to be able to cause the loss of more than 95% of mitochondrial trancriptional capacity (Kristal *et al.* 1997) and several data indicate that in the hepatocytes of diabetic animals the production of reactive oxygen species in mitochondria is strongly stimulated (Karagezian *et al.* 1990; Goldkorn *et al.* 1998). On the other hand, oxidation products occurring in diabetes may be involved in subtoxic processes at the level of signal transduction: indeed, several oxidants stimulate tyrosine as well as serine/threonine phosphorylation (Pugnaloni *et al.* 1999, Goldkorn *et al.* 1998; Clerk *et al.* 1998; Bize *et al.* 1998), which is known to be a key step in signal transduction processes.

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Several factors are involved in the pathological consequences of diabetic hyperglycemia: among those are oxidative stress, the direct toxic effect of increased glucose levels, and the bioenergetic deficits occurring as a consequence of the metabolic derangement.

In particular, among other consequences, it is known that oxidative stress promotes an accumulation of intramitochondrial Ca^{2+} which determines, or contributes to, the production of mitochondrial DNA damage (Low *et al.* 1997; Cobo *et al.* 1997).

Altered Ca^{2+} homeostasis and impaired gene expression patterns may play a crucial role in the clinical onset of several diseases, and various mitochondrial dysfunctions can have their key factors in lowered electron transport rate and low mitochondrial energy coupling (Olson *et al.* 1998; Crane *et al.* 1994; Matsuoka *et al.* 1997; Guidarelli *et al.* 1997).

Defective mitochondrial electron transfer enhances the NADH/NAD+ cytoplasmic ratio, an event that has important metabolic consequences, first of all an increased lactate production; the importance of the pyridine nucleotide redox equilibrium in relation to diabetes is even clearer if one considers that reduced redox capacity has been reported in the mitochondria of glucose-stimulated Langerhans islets (Matschinnsky 1996; Ramirez *et al.* 1996). Moreover, glucose-stimulated insulin release has been seen to stimulate in turn mitochondrial dehydrogenase activity and translocation of Ca^{2+} from mitochondrion to cytosol (Ramirez *et al.* 1995; Moran *et al.* 1997; Salceda *et al.* 1998).

The aim of the present work was to assess quantitatively by an immuno-electron microscopic study the presence of phosphorylated tyrosine in the nucleus, cytoplasm, mitochondria and cell membrane (in parallel with cell membrane NADH oxidase activity, a cell membrane redox marker) in lymphocytes from IDDM patients and normal individuals in order to seek possible relationships with the glycemic status.

Given the firm evidence for mitochondrial alterations in IDDM lymphocytes (Vidal *et al.* 1997; Novials *et al.* 1997), the present study of diabetic patients was performed in an easily accessible cell population, i.e. peripheral blood lymphocytes, which are considered a useful tool to monitor IDDM disease behaviour, to obtain crucial data on the energy status of the immunological compartment, as well as to monitor the parameters related to ischemia and abnormal blood viscosity (Morigi

et al. 1998; Ceriello *et al.* 1998; Victor *et al.* 1985), which are closely connected with the function and metabolic derangement of IDDM.

MATERIALS AND METHODS

Materials

Monoclonal anti-phosphotyrosine antibody (4G10) was purchesed from UBI (Lake Placid, NY), 12-nm gold particle-conjugated secondary antibody from Jackson Immuno Research (West Grove, PA), and L.R. White embedding resin from Polyscience (Warrington, PA).

All the other reagents were of molecular or analytical grade.

Patients and Methods

Lymphocytes were obtained by centrifugation from 40 ml of peripheral venous blood drawn from 11 IDDM patients and 9 healthy controls aged 5 to 19 years who gave their informed consent to participate in the study. Lymphocytes were divided into two aliquots: one was used immediately (T^0) and the other after 30 min at 4°C (T^{30}) for parallel biochemical and immunomorphological studies.

Owing to the need for testing blood samples of at least 40 ml and to the compromised state of some patients, in 4 cases the same lymphocyte aliquots were studied for both series of experiments (Table 1).

a- Morphology and Immunocytochemistry

For the morphological study, pellets of lymphocytes were fixed in 1% glutaraldehyde in 0.1M phosphate buffer (PB) for 1 h at 4°C, washed overnight in 0.15M PB, dehydrated in ethanol and embedded in resin. Ultrathin sections (90 nm) were cut and mounted on nickel grids. The immunocytochemical study of tyrosine phosphorylation (Alessandro *et al.* 1997; Pugnaloni *et al.* 1999) was performed on post-embedded material using a primary anti-phosphotyrosine monoclonal antibody, 4G10, (1:100) in Tris-HCl (50 mM Tris, 0.14 M NaCl, pH 7.6) containing 0.1% bovine serum albumin (BSA). Sections were then washed and incubated with goat anti-mouse (GAM)IgG conjugated with 12-nm colloidal gold particles (1:10) in 20 mM Tris-HCl, pH 8.2, containing 0.1% BSA for 1 h at room temperature. Samples not incubated with the primary antibody were

Table I
Lymphocytes from control subjects and from IDDM patients: evaluation of phosphotyrosine expression (4G10 MoAb 1:100. No gold particles/(mm^2 o (mm*) in cells used at T^0 and T^{30} (mean values ± sd)

Cases	Cytoplasm	Nucleus	Total Cell Labelling	Cell Membrane*	Mitochondria
Control subjects T^0	10.07 ± 5.80	5.33 ± 4.15	8.31 ± 5.01	1.33 ± 1.01	19.03 ± 10.86
Control subjects T^{30}	4.59 ± 2.32	2.38 ± 2.19	3.45 ± 1.78	0.93 ± 0.48	5.79 ± 3.04 **
IDDM patients T^0	9.68 ± 5.74	5.34 ± 5.20	8.72 ± 7.23	1.97 ± 3.76	17.34 ± 12.89
IDDM patients T^{30}	6.50 ± 4.73	3.49 ± 2.56	5.25 ± 3.41	0.89 ± 0.42	13.08 ± 15.02

** p< 0.05 - Mann Whitney U test

used as controls and did not stain (data not shown). Grids were counterstained with aqueous uranyl acetate and lead citrate.

Morphometric evaluations

Specimens were observed and photographed using a Philips CM10 (Eindhoven, The Netherlands) transmission electron microscope at 80 KW. For each sample, 20 images at 18,000X magnification which included membrane, cytoplasm and nucleus were selected and evaluated with an image analyser (Lucia-Nikon, Italy) using an interactive software. Gold-particle density was expressed as absolute density, i.e. as the number of gold particles counted per μm^2 or μm in different cellular districts (cytoplasm, nucleus, mitochondria and cell membrane), or as total cell labelling per μm^2 of total cell surface. In the case of mitochondria, labelling density was referred to the total mitochondrial area of each cell. Data distribution was assessed and is shown graphically in Fig. 3. Because of the high standard deviation (sd) values observed in all cellular districts (mainly in mitochondria), statistical evaluations were performed using the Mann Whitney U non-parametric test.

b-Biochemistry. Plasma membrane NADH oxidase

Intact lymphocytes from both IDDM patients and controls were used for this study. Plasma membrane oxidoreductase activity was assayed using either ferricyanide (K$_3$Fe(CN)$_6$) or dichlorophenol indophenol (DCIP) as acceptors (Vaillant *et al.* 1996). The electron donor under the conditions used was endogenous NADH. The activity was assayed in the presence of mitochondrial inhibitors to avoid possible interference with mitochondrial oxidative activities.

For NADH-ferricyanide reductase activity 0.5-1·10^6 cells were washed in 500μl phosphate buffer saline (PBS) (0.15 M NaCl, 20mM Na2HPO4 ·H$_2$0, pH 7.4) and 2 mM KCN, pH 7.4, and resuspended in 1ml PBS containing 10 mM glucose, 1 mM pyruvate, 1μg/ml rotenone, 1 g/ml antimycin A, 1μg/ml myxothiazol, 2mM KCN, for 10 min at 37°C. Cells were then added to 1.5 ml reaction mixture containing PBS (NaCl 0,15 M, 20mM Na$_2$HPO$_4$· H$_2$O$_2$), KCN 2 mM, pH 7.4 and 250μM K$_3$Fe(CN)$_6$.

Ferricyanide reduction was followed at 420-500 nm by SIGMA-ZWS II Zweiwellenlaengen-spectral photometer, for 4 min at 37°C; the molar extinction coefficient used was of 1,000 M-1cm-1.

For NADH-DCIP reductase activity, cells treated as above were added to 1.5 ml PBS reaction mixture containing 2mM KCN, pH 7.4 and 20μM DCIP. In this case, DCIP reduction was monitored at 600-700 nm for 2 min; the molar extinction coefficient used was 21 mM^{-1} cm^{-1}.

RESULTS

Morphological and immunocytochemical evaluations

Controls:T^0 (Fig. 1, a). At T^0, values were fairly inhomogeneous, with high sd, although they never exceeded the mean values. This finding could be related to the inhomogeneity of the lymphocyte pop-
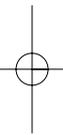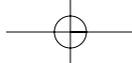
Fig. 1 - Immunoelectron micro-
scopy of phosphotyrosine in iso-
lated control lymphocytes pre-
pared as described in Materials
and Methods. Ultrathin sections
were incubated with antiphos-
photyrosine and detected with
immunogold reagent. Transmis-
sion electron micrographs were
taken at 39,500X final magnifi-
cation. M = mitocondria. a) Con-
trol control cells at T^{30}.

ulation itself and therefore to the different expression
of phosphorylated tyrosine by the different types of
lymphocytes. The numerical density of gold particles
per m2 was 10.07 ± 5.80 in the cytoplasmic district,
5.33$\pm$ 4.15 in the nucleus, 19.03 ± 10.86 in mito-
chondria and 8.31 ± 5.01 on the whole cell surface. At
the level of the plasma membrane, numerical density
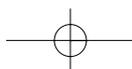per m of membrane length was 1.33 ± 1.01.

Table II

Lymphocytes from control subjects and from IDDM patients: evaluation of phosphotyrosine expression (4G10 MoAb 1:100. No gold particles/mm^2 or (mm*) after 30 min. Values are expressed as percent of T^0 condition (mean values ± sd)

Cases	Cytoplasm	Nucleus	Total Cell Labelling	Cell Membrane*	Mitochondria
Control subjects	42.90	44.65	41.52	69.92	30.42
IDDM patients	60.74	65.48	63.18	66.92	68.73

Controls: T^{30} (Fig. 1, b). Thirty minutes after collection, values decreased in all cytoplasmic districts: gold-particle density per μm^2 was 4.59 + 2.32 in the cytoplasm (42.90% of T^0), 2.38 ± 2.19 in the nucleus (44.65%), 5.79 ± 3.04 in mitochondria (30.42%), and 3.45 ± 1.78 on the whole cell surface (41.52%); finally, in the plasmalemma, numerical density per m of membrane was 0.93 ± 0.48 (69.92% of T^0).

IDDM: T^0 (Fig. 2a). In IDDM patients, values were also inhomogeneous with high sd in the same cell districts: cytoplasm (9.68 ± 5.74), nucleus (5.34 ± 5.20), mitochondria (17.34 ± 12.89), total cell surface (8.72 ± 7.23) and cell membrane (1.97 ± 3.76).

IDDM: T^{30} (Fig. 2,b). Also in this case, values decreased from T^0 to T^{30}. Mean labelling and percent labelling reductions were, respectively, 6.50 ± 4.73 and 60.74% in the cytoplasm, 3.49 ± 2.56 and 65.48% in the nucleus, 13.08 ± 15.02 and 68.73% in mitochondria, 5.25 ± 3.41 and 63.18% on total cell surface and 0.89 ± 0.42 and 66.92% in the plasmalemma. The reduction in phosphotyrosine expression was thus less rapid in the lymphocytes of IDDM patients. This was especially evident in mitochondria, even though values were less homogeneous.

In the mitochondria, significant differences (p<0.05) were found between the lymphocytes of control and diabetic subjects at T^0 and T^{30}, respectively. In this cellular district, control subjects at T^{30} showed a numerical density of 5.79 ± 3.04 per μm^2, with a 30.42% reduction with respect to T^0.

The trend of phosphorylation in the different conditions was represented with a diagram (Fig. 3), and mean values, with the corresponding sd, were calculated for the various samples to evidence their homogeneity or lack of it (see Materials and Methods).

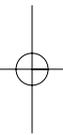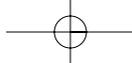**Biochemical Aspects of Plasma Membrane NADH-oxidase activity (Table III)**

An increase in NADH→DCIP activity (p<0.005), index of a higher redox potential inside cells, was observed in patients (2.69 ± 0.59 nmol · min^{-1} · 10^{-6} cells) with respect to controls (1.97 ± 0.51 nmol · min^{-1} · 10^{-6} cells). No difference between the two groups was detected in NADH→ferricyanide activity (0.62 ± 0.24 nmol · min^{-1} · 10^{-6} cells in IDDM, and 0.73 ± 0.37 nmol · min^{-1} · 10^{-6} cells in controls) probably due to the low efficiency of this electron acceptor.

DISCUSSION

The main underlying metabolic cause of diabetes is the failure of pancreatic beta cells to supply insulin in amounts sufficient to meet the body's requirements, resulting in hyperglycemia. Juvenile (type 1)

Table III

Plasma membrane NADH oxidase activity in lymphocytes (mean values ± sd)
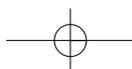
	Control Subjects (n=11)	IDDM Patients (n=13)	
	(nmol · min^{-1} · 10^{-6} cell)		
NADH→DCIP	1.97 ± 0.51	2.69 ± 0.59	p <0.005
NADH→K$_3$Fe(CN)$_6$	0.73 ± 0.37	0.62 ± 0.24	p = n.s.

Fig. 2 - Immunoelectron microscopy of phosphotyrosine in isolated cells at T^0, b) lymphocytes from IDDM patients treated as control samples. M = mitochondria. a) cells at T^0, b) cells at T^{30}.

diabetes is determined by the immune destruction of beta cells. Adult-onset (type 2) diabetes, which accounts for 90% of cases of diabetes, is characterised by a partial deficit in insulin production associated with resistance by peripheral tissues to the

action of the hormone. Polygenic traits contribute to the pathogenesis of both types of diabetes. However, a specific form of type-2 diabetes -Mody- is monogenic due to inactivating mutations in genes that are critical for normal beta-cell function
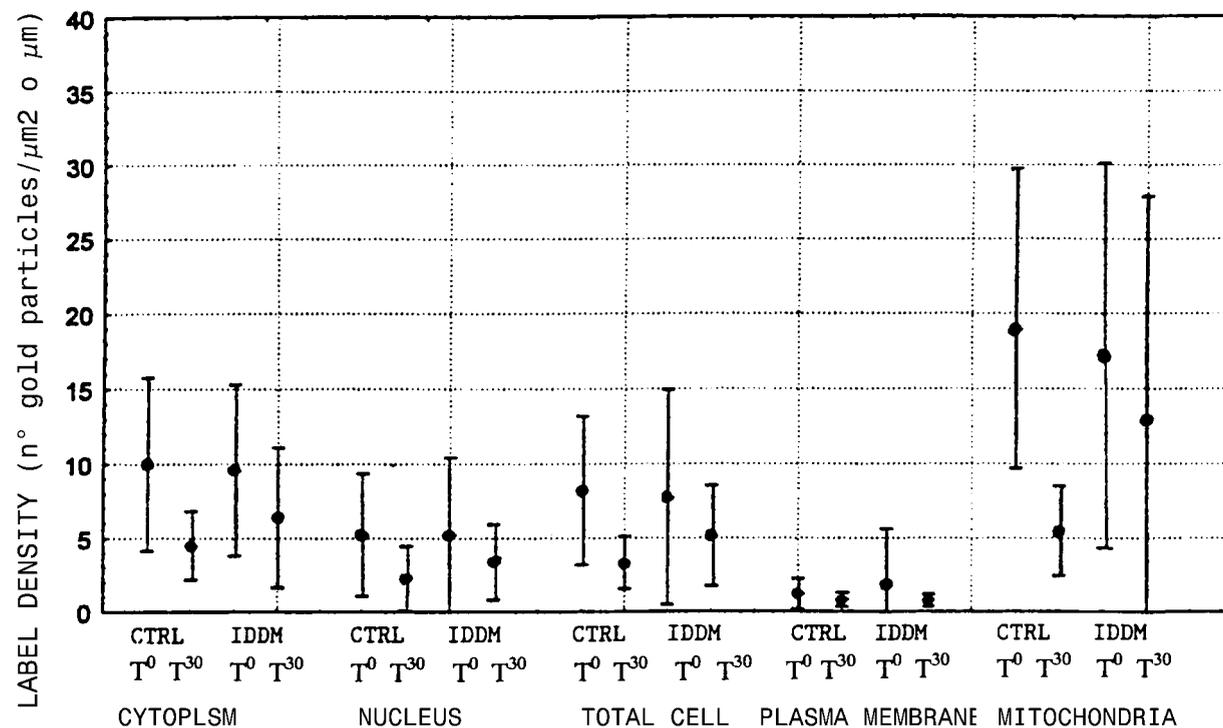
Fig. 3 - Graphic representation of the distribution of morphometric data. High sd values were observed in all cellular districts, mainly in mitochondria. Standard deviations from mean values in control mitochondria at T^0 and T^{30} were not overlapping, with statistically significant differences of the data ($p<0.05$).

(Chaturvedi *et al.* 1997; Rabinovitch *et al.*1998; Miller *et al.* 1999).

At the peripheral level, free-radical overproduction contributes to the tissue damage induced by acute hyperglycemia and the administration of molecules with anti-oxidant properties increases tissue resistance to the lipid peroxidation triggered by acute hyperglycemia.

Hyperglycemia may also induce a parallel increase in circulating ICAM through oxidative stress. ICAM is among the most important intercellular adhesion molecules involved in diabetes, even if it is also associated with different pathologies such as atherosclerosis, and it is expressed in activated circulating mononuclear cells, including T cells (Ceriello *et al.* 1998).

These functional data agree with our findings of higher levels of tyrosine phosphorylation -expression of upregulation- in the lymphocytes of IDDM hyperglycemic patients compared with healthy subjects.

Normal circulating mononuclear cells are known to respond to physiological concentrations of insulin by rapidly increasing the amount of glu-

cose that is transported into the cell. The same cells from IDDM insulin-dependent subjects show less capacity for glucose transport both in basal conditions and following insulin stimulation (Estrada *et al.* 1994).

Insulin stimulates glucose transport via the translocation of the glucose transporters (Glut-1, Glut-3 and Glut-4) from intracellular stores to the plasma membrane. But, for this process to work, an intact actin network and phosphatidyl-inositol 3-kinase activity are necessary (Muller *et al.* 1998; Kao *et al.* 1999). Insulin also stimulates autophosphorylation of the insulin receptor on tyrosine (Carter *et al.* 1996).

Tyrosine phosphorylation at the protein level plays a vital role and is a primary event in signal transduction for the regulation of glucose metabolism (Carter 1996, Pugnaloni *et al.* 1999). In lymphocytes too, antigens or cytokine receptors induce rapid tyrosine phosphorylation at the level of the receptor subunit, of different membrane proteins and of other signal components that are necessary to maintain a correct functional state in these cells (Saad *et al.* 1997; Naz 1996; Subrah-

manyam *et al.* 1991). In human fibroblasts it has long been known that 50-kDa tyrosine-phosphorylated protein bands can be observed also in purified mitochondria (Piedimonte *et al.* 1986).

Similarly, rat liver mitochondria show protein phosphorylation on specific bands from 47 to 36 kDa (Backer *et al.* 1986). In spermatozoa, tyrosine phosphorylation has been detected immunocytochemically at the mitochondrial level (Travis *et al.* 1998).

At the time of collection the tyrosine phosphorylation level was not specifically different in any cellular district of IDDM patients with respect to age-matched controls; however, the morphometric evaluations performed on healthy and IDDM lymphocytes showed that 30 minutes from collection phosphotyrosine levels decreased in all cellular districts possibly as a result of activation of tyrosine phosphatases. This reduction was greater in control lymphocytes, except in the plasma membrane, where the reduction in phosphotyrosine levels was greater in IDDM cells.

In particular at the mitochondrial level, phosphotyrosine values at T^{30} decreased only marginally in the lymphocytes from IDDM patients, whereas at T^0 phosphoprotein values were similar in the two groups of subjects. This finding could be related to the greater activation state of lymphocytes from IDDM patients, more exposed to oxidative-stress stimulation and to altered extracellular-glucose input, maintaining a further steady-state level of tyrosine phosphorylation with time.

It is known that also for lymphocytes, triggering and activation signal-transduction pathways involve tyrosine phosphorylation and dephosphorylation (respectively via kinases and phosphatases).

Several reports, including observations made in our lab (unpublished), are in line with this hypothesis (Hanna *et al.* 1997; Honeyman *et al.* 1998). This activation state should be interpreted in the light of biochemical data.

We found indications of a significant derangement of mitochondrial function in the lymphocytes of diabetic patients.

The mitochondrial membrane potential did not appear to be altered in IDDM cells at T^0, possibly by virtue of effective respiratory-chain functioning, which usually tends to compensate the membrane potential (unpublished data).

However, the *in-vitro* addition of increasing concentrations of an uncoupling agent, FCCP, caused a modification in the slope of the regression line, indicating the difficulty of the respiratory chain of diabetic mitochondria to maintain the stationary steady state of the proton electrochemical gradient (D'Aurelio *et al.* 1998).

The increase in NADH oxidase at the level of the plasma membrane observed in our IDDM lymphocytes is an expression of an increase of cytoplasmic NADH as a result of impaired mitochondrial activity and should be considered as a compensatory mechanism to release excess cytosolic reducing power (Morre *et al.* 2000). This concept correlates well with i) the presence of an altered plasma micro-environment in IDDM patients, with free radicals stimulating oxidizing activity, and ii) the finding that the redox state of quinone oxidase controls tyrosine kinase via H^2O^2 generation or through conformational redox-inducing modifications (Greiber *et al.* 1998). The plasma membrane of eukaryotic cells contains a NADH oxidase which transfers electrons across the membrane (Morre et al 1998; Van Duijn *et al.* 1998; Kishi *et al.* 1999). This oxidase is controlled by hormones (Chueh *et al.* 1997), growth factors (Morre *et al.* 1997) and other molecules (Dai *et al.* 1997) which bind to receptors in the plasma membrane, so it is also linked to the ligand-dependent tyrosine phosphorylation cascade. Oxidants, moreover, activate growth-related signals such as calcium mobilization, while coenzyme-Q analogues reversibly inhibit both growth and transmembrane electron transport (Brightman *et al.* 1992).

In conclusion, literature data correlate well with the immunohistochemical and biochemical findings of our study, which also seems to point to a possible role for mitochondria as signal transducers in cellular metabolic activities, especially considering the relevance of mitochondrial signals, such as Bax transfer in cytochrome-c release in the signal transduction involved in cell apoptosis (Pedersen, 1999).

Signal transduction may thus represent one of the most easily hit targets in the course of IDDM development.

ACKNOWLEDGMENTS

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