EFFECT OF DIOXIN ON NORMAL AND LEUKEMIC HUMAN HEMATOPOIETIC CELLS

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Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) arises from chlorination of phenolic substrates or from partial combustion of organic materials in the presence of chlorine sources[1]. TCDD has a large number of biological effects such as long-lasting skin disease[2], cardiovascular disease, diabete and cancer[3]. TCDD is the prototypical agonist of the aryl hydrocarbon receptor (AhR)[4], a member of the erb-A family that also includes the receptors for steroids, thyroid hormones, peroxisome proliferators and retinoids. When bound to dioxin, the AhR can bind to DNA and alter the expression of some genes including cytokines and growth factors.

In this study, we analyzed the effect of escalating doses of TCDD on human $CD34^+$ progenitor cells from the leukapheresis of normal donors stimulated with G-CSF as well as the human myeloid leukemic cell lines HL60 (promyelocytic leukemia) and K562 (chronic myelogenous leukemia). The possibile specific modulation of gene expression induced by the TCDD exposure was then tested by means of microarray analyses.

Methods and Materials

Hematopoietic progenitor $CD34^+$ cell separation: the $CD34^+$ haematopoietic progenitor cells were separated (after informed consent) from leukapheresis of normal donors stimulated with G-CSF using the immunonomagnetic method MACS Miltenyi. The bone marrow specimens were layered on a Ficoll-Paque gradient (specific gravity 1.077 g/ml; Nycomed Pharma AS, Oslo, Norway) in order to separate the LD-MNC; the samples were then washed twice in Hanks' balanced salt solution (HBSS). The LD-MNC were incubated for 15 minutes at 4°C with the QBEND10 monoclonal antibody directed against the CD34 antigen. The cells were then washed and incubated for 15 minutes at 4°C with immuno-magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) against QBEND10. For the flow cytometry analysis, CD34-phycoerythrin (CD34-PE) conjugated antibody (HPCA-2, Becton Dickinson, Mountain View, CA, USA) was added to the cells for 15 minutes at 4°C. At the end of the separation, the cells were counted and assessed for viability by means of trypan blue dye exclusion; their purity was determined by means

of flow cytometry analysis. All of these steps were performed under aseptic conditions. The mean percentage of purity of the $CD34^+$ cells after immuno-magnetic separation was 90.6%.

Cells: the human promyelocytic leukaemia HL60 and chronic myelogenous leukemia K562 cell lines were obtained from Interlab Cell Line Collection (ICLC, Genova, Italy). All the cell lines were grown in RPMI + 10% FCS at 37°C at densities ranging between 2 and 3 x 10^5 cells per ml.

Cytotoxicity on clonogenic assays (CFU-GM): the effect of TCDD on cell growth was evaluated on the capability of the treated cells of colony formation. The clonogenic assays were carried out by plating $5x10^3$ $CD34^+$ cells or $1x10^3$ HL60 or K562 cells in a methylcellulose culture medium (MethoCult 4434, StemCell Technologies Inc., Vancouver, Canada) containing 0.9% methylcellulose in Iscove's MDM, 30% fetal bovine serum, 1% bovine serum albumin, 10^{-4} M2-Mercaptoethanol, 2 mM L-glutamine, 50 ng/ml rh Stem Cell Factor, 10 ng/ml rh GM-CSF, 10 ng/ml rh IL-3, 3 Units/ml rh Erythropoietin with different doses of TCDD. The cells were pretreated for 12-24 hours with TCDD and then seeded in the semisolid medium. Triplicate dishes were incubated at 37°C in a 5% CO_2 fully humidified atmosphere. The aggregates of ≥ 40 and < 40 cells were respectively scored as colonies and clusters after 14 days of culture.

Microarrays Analysis: Atlas 1.2II cDNA expression arrays from Clontech Laboratories (Palo Alto, CA) were chosen. These arrays consist of 1176 human cDNA fragments, organized into broad functional groups. A complete list of the genes included on the membranes is available on the Clontech Web site (http://www.clontech.com). All cDNAs printed on the arrays have been sequence-verified by the company. The microarrays were screened using radiolabeled cDNA generated from total poly$(A)^+$ RNA from $CD34^+$ cells as well as HL60 and K562 cells lines cultured in the presence or absence of 20 nM TCDD for 12-24 hrs. Differential expression was arbitrarily defined as any gene over- or down- modulated in the cell line by a ratio greater than 2 at any signal intensity.

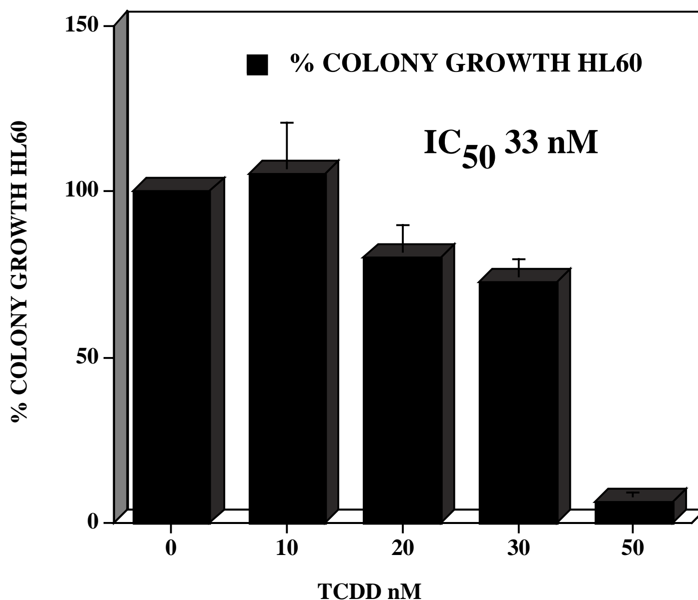
Results and Discussion

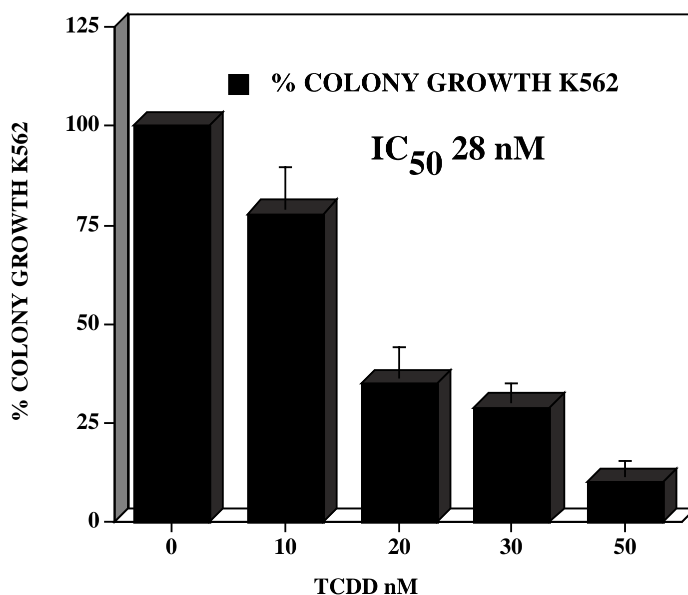
Cytotoxicity: the dioxin dose capable of inhibiting the growth of 50% of the colonies in semisolid medium (IC_{50}) was 33 nM for HL60, 28 nM for K562 and 20 nM for the $CD34^+$ cells (Figure 1). The IC_{50} was calculated with CalcuSyn computer program.

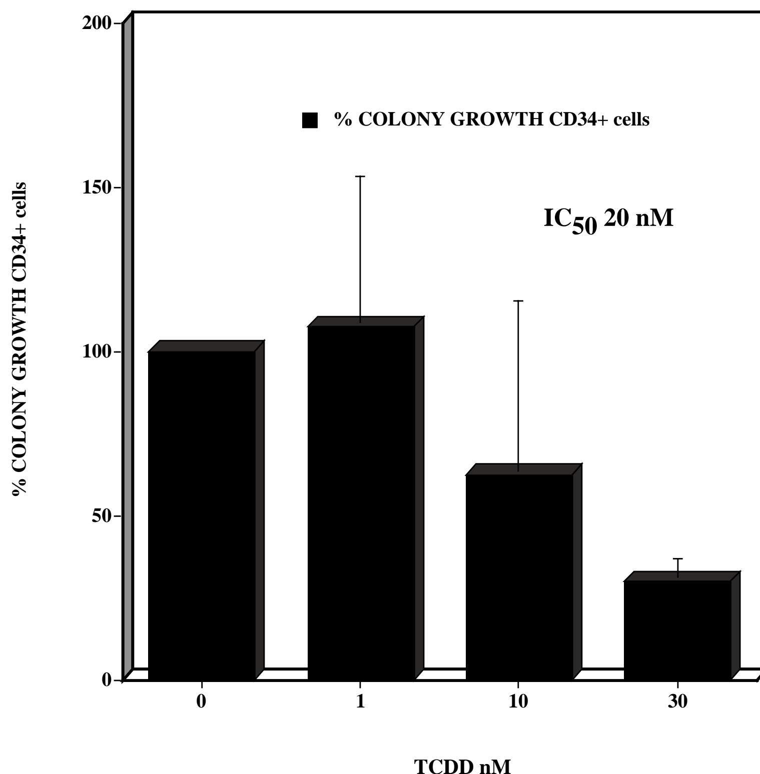
Gene expression modulation: the possibile specific modulation of gene expression was tested by means of microarray analyses of the HL60, K562 and $CD34^+$ cells before and after exposure to 20 nM of TCDD. We identified a series of genes modulated by TCDD exposure. Among the up-regulated genes were LUCA15 putative tumor suppressor, cyclin-D binding Myb-like protein (hDMP1), MAPK/ERK kinase 6, ras-related protein RAB2, RalB GTP-binding protein, transcription factor ZFM1, BRCA1-associated ring domain protein (BARD1). Among the down-regulated genes we found c-myc, TNF alpha precursor, ICAM1, MIP1-beta, interferon gamma-induced protein precursor (gamma-IP10), granulocyte-macrophage colony stimulating factor (GM-CSF), monocyte chemotactic protein 1 precursor (MCP1). All of these genes are variously involved in the processes of proliferation, differentiation and transformation in haematological and tumoral cell models. Our data on TCDD biological effect on the $CD34^+$ normal progenitor cells, together

with the epidemiologic data of increased hematologic cancer risk in the population exposed accidentally to the substance, suggest a pathogenetic role of TCDD in the neoplastic transformation of hemopoietic stem cells and provide a molecular basis for this hypoyhesis.

Figure1: clonogenic growth in semisolid medium after treatment with TCDD 20 nM of: A) HL60 cell line, B) K562 cell line and C) $CD34^+$ immuno-magnetically separated human normal progenitor cells. All data are expressed as mean percentage of the untreated control. Each histogram represents the mean ± SD of three independent experiments.







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