Leukemic Cell Differentiation and the Signal Transduction System

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Differentiation and proliferation uncoupling anomalies make an important contribution to the leukemogenic process and the events leading to this catastropic process have not yet been clarified. The triggering of uncontrolled proliferation and the disturbance of differentiation occur by the complex cascade of signal pathways. To enlighten the mechanisms related to leukemogenesis, model cell lines are popular tools of investigation. Among these cell lines, myeloid leukemia cell line, HL-60, is a very good and established model of leukemia[1].

HL-60 cells differentiate along the granulocytic or the monocytic pathway when treated with inducer molecules. This myeloid leukemia cell line differentiates to granulocytic cells upon all-trans retinoic acid and dimethyl sulfoxide stimulation. It differentiates to monocytic cells upon stimulation with 1,25 $(OH)_2D_3$, and to macrophage-like cells upon stimulation with phorbol ester derivatives. This characteristic feature of HL-60 cells attracted researche'rs interest, and this cell line has become a frequently described model system for cell differentiation in vitro [2].

1,25 $(OH)_2D_3$ induces human myelogenous leukemic HL-60 cells, as well as normal human bone marrow cells, to differentiate terminally into mature monocytes. Differentiation with this agent is coupled to inhibition of proliferation. The intracellular mechanisms leading to terminal differentiation have yet to be clarified. Because retinoic acid has proved to be effective in differentiation therapy of acute promyelocytic leukemia, analogues of 1,25 $(OH)_2D_3$ are also currently being investigated as putative differentiation-inducing agents to treat patients with leukemia[3,4].

ATRA induces granulocytic differentiation in cultured leukemic HL-60 cells and is clinically effective as a differentiation therapy in inducing high remission rates in patients with acute promyelocytic leukemia. The biologic effects of ATRA appear to be mediated through a number of closely related nuclear retinoic acid receptors that possess discrete DNA-binding and retinoic-acid binding domains[5]. Although the exact mechanism of the ATRA-induced granulocytic differentiation remains a mystery, protein phosphorylation/dephosphorylation is also thought to be a regulatory device eminently suited for the control of differentiation process. The levels of both protein kinase C activity and the expression of its isoforms have been shown to increase during HL-60 cell differentiation, induced by dimethyl sulfoxide and ATRA[7].

Methylprednisolone is also a member of the steroid hormones, including retinoic acid and vitamin D3, and act by binding to members of the zinc-finger con-

taining the superfamily of nuclear hormone receptors. These receptor proteins bind directly to specific DNA recognition sequences in the promoter region of target genes, resulting in the alteration of transcription initiation rate [8]. High-dose methylprednisolone (HDMP) treatment has been shown to induce in vivo differentiation of myeloid leukemia cells to mature granulocytes in patients with acute promyelocytic leukemia and other subtypes of acute myeloblastic leukemia[9]. A recent study from our laboratory showed that methylprednisolone suppressed the proliferation of HL-60 and K 562 cells (Ph positive transformed CML acute myeloid leukemia cell line), and induced these cells to differentiate terminally into granulocytic and monocytic cells, respectively[10]. Methylprednisolone induced differentiation for both cell lines was dose dependent at concentrations of methylprednisolone from 0.8 to 2 mM. In certain concentration intervals, K 562 cells were found to be more resistant to differentiation as compared to HL-60 cells.

Lately, arsenic trioxide has been described in the treatment of acute myeloid leukemia. A partial differentiation of APL cells and down regulation of the fusion protein, PML /RARa and bcl-2, have been seen in the APL cells, in vitro and in vivo. Although, arsenic trioxide has been known for a long time, apoptosis and the differentiation of APL cells is a novel observation[11]. We have also demonstrated the partial differentiative effects of arsenic trioxide on HL-60 and K 562 cells (unpublished data).

Protein phosphorylation is a primary event that mediates the signal transduction systems that controls processes such as cell proliferation and differentiation. Kinases and phosphatases modulate the protein phosphorylation "status" of a cell, which in turn directly regulates numerous fundamental biological phenomenon such as cell division and development.

In this review, we will try to emphasize the dephosphorylation cascade and the changes that occur along the different pathways of differentiation that was enlightened to an extent.

PROTEIN PHOSPHATASES

These enzymes, which mediate the dephosphorylation reactions on one side of the phosphorylation equilibrium of the cell, are grouped as tyrosine phosphatases and serine/threonine phosphatases with respect to the amino acids on which they exert their effects as substrate proteins. These enzymes are built of catalytic and regulatory subunits. The major serine/threonine protein phosphatase catalytic subunits of mammalian cells comprise four forms which have been designated as: protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), protein phosphatase 2B (PP2B, calcineurin), and protein phosphatase 2C (PP2C). This is based on a classification system proposed by Cohen. PP1 and PP2A do not have an absolute requirement for divalent cations, whereas PP2B and PP2C are Ca^{2+}/calmodulin-and Mg^{2+}-dependent, respectively. Okadaik acid (OKA), a recently discovered tumor promoter, is a polyether fatty acid with IC_{50}s of 60-200nM for PP1 and 1-10 nM for PP2A, therefore it can distinguish between PP1 and PP2A. Calyculin A (Cal-A) is a spiroketal fatty acid with IC_{50}s of 2 nM for PP1 and 1 nM for PP2A. While the regulatory subunits of PP2A have been purified and cloned, much less is known of the regulatory subunits of PP1. Three different, but highly similar, isoforms of PP1 catalytic subunits (PP1a, PP1g, PP1d), with a 85-95% amino acid sequence identity, have been identified by cDNA cloning, and their mRNAs are expressed in a wide range of mammalian tissues. PP1 isoenzymes have been identified in both soluble and particulate cellular fractions, and in various forms. The glycogen-associated form of PP1, termed PP1G, consists of the catalytic subunit complexed to a Mr 124,000 G subunit which targets PP1 to glycogen particles. Recently, a myosin-bound PP1 holoenzyme in smooth muscle cells was isolated and was found to consist of a catalytic subunit Mr 130,000 and a Mr 20,000 regulatory subunit[12,13].

The PP2A catalytic subunit (PP2A-C,Mr 36,000) is mainly present as a holoenzyme, forming a heterotrimer with a (Mr 65,000) regulatory and different B regulatory subunits, the basic form is the PP2A-C/A complex and the B subunit is associated with the dimer through A subunit. The A and B regulatory subunits were shown to modulate the phosphatase activity of the PP2A-C in vitro. The cDNA clones for the two respective isoforms of PP2A-C and A subunit, PP2A-Ca and PP2A-Cb and Aa and Ab, respectively, have been isolated from several animal species. Based on biochemical evidence, the B subunits of PP2A holoenzyme is comprised of several distinct families of proteins of B (Mr 55,000), B' (Mr 54,000), B'' (Mr 74,000) and Mr 72,000. Three cDNAs for isoforms of the B (Mr

55,000) subunit (a,b,g) have been cloned from human, rabbit, rat, and yeast libraries. A, B, and PP2A-C subunits show a high degree of sequence conservation among species. Although the effect of the regulatory subunits of PP2A on the substrate specificity has clearly been demonstrated in vitro, the roles of individual regulatory proteins in the regulation of metabolism, growth, differentiation, and development remain largely unknown[13].

Calcineurin (PP2B), has been identified in various tissues, and was originally thought to occur primarily in nerve tissue. The calcineurin holoenzyme purified from mammalian cells is a heterotrimeric complex consisting of a large 60 kD catalytic subunit, calcineurin A (CNA), 19kD Ca^{2+}-binding regulatory subunit, calcineurin B (CNB) and calmodulin, an 17 kD Ca^{2+}-receptive protein. The A subunit binds calmodulin, whereas the B subunit attaches to four molecules of Ca^{2+}. Calcineurin regulatory subunit, CNB, is the key site of association for both the cyclosporin A (CsA) and FK506-immunophilin complexes. Calcineurin is incapable of functioning as a phosphatase in vitro when calmodulin or CNB is absent. Although sequences of CNB and calmodulin are related, neither of these two proteins can complement the other's absence. Calcineurin is required in activation and proliferation of T lymphocytes to activate the NF-AT transcription factor involved in the regulation of the interleukin-2 (IL-2) receptor gene and NF-KB serves as a molecular switch[14].

In mammalian cells, two isoenzymes of PP2C (PP2Ca and PP2Cb) have been cloned, and the two isoforms display an overall homology of 76%. However, little is known about the physiological functions, mainly because of the unavailability of specific inhibitors of PP2C. It has been reported that the expression of the PP2C gene is enhanced during MyoD1-induced myogenic differentiation of embryonic mesenchymal cells[15].

Monocytic Differentiation and Dephosphorylation Dynamics[13,15].

Cal-A, a potent inhibitor of PP1 and PP2, augments 1,25 $(OH)_2D_3$-induced monocytic differentiation of HL-60 cells and this finding is evidence for the involvement of protein phosphatases. The treatment of HL-60 cells with 1,25 $(OH)_2D_3$ led to a decrease in the cytosolic PP1-like activity with a concomitant increase in the PP1-like activiy of membrane and nuclear fractions. A reduction of the cytosolic PP1-like activity correlated with the increased expression of certain differentiation markers related to monocytes. The subcellular redistribution of PP1-like activity induced by 1,25 $(OH)_2D_3$ was mainly due to the selective translocation of the cytosolic PP1g and PP1a proteins to membrane and nuclear fractions, whereas the distribution of PP1d and PP2A was relatively constant. Interestingly, the translocation of PP1g was more rapid than that of PP1a, hence the individual PP1 isoenzymes might be differently regulated. PP1 was shown to be colocalized with chromosomes in mammalian cells at mitosis. The intracellular distribution of PP1 activity may be an important aspect of the regulation related to the differentiation of HL-60 cells. The induction of c-fos mature transcripts, and an increase in AP-1 activity, have been noted in cases of 1,25 $(OH)_2D_3$-induced inhibition of proliferation and monocytic differentiation. 1,25 $(OH)_2D_3$ was found to cause a decrease in c-myc mRNA expression in HL-60 cells. These nuclear events may be related to the increased PP1g activity in the nucleus, facilitating the activated state of definite transcription factors, including the 1,25 $(OH)_2D_3$ receptor, and affecting the processes of differentiation. It has been shown that PP1 activity is associated with glycogen particles and SR membranes and is enriched in other particulate fractions and organelles such as microsomes, ribosomes, nuclei, and myofibrils. The increase of PP1g and the PP1a protein in the membrane fraction, translocating from the cytosol fraction, may be indicative of the changing targeting specificity. The decrease in immunoreactive PP1g and PP1a isoforms of cytosol fraction during 1,25 $(OH)_2D_3$-induced differentiation was not associated with a modified expression of PP1g and PP1a genes. Thus, the modulation of PP1 isoenzymes may be involved in the regulation of 1,25 $(OH)_2D_3$-induced differentiation. PP1 consists of a multimeric structure composed of a catalytic subunit complexed to a number of regulatory components that play important roles in regulating phosphatase activity or targeting it to specific subcellular locations. The free catalytic subunit has not been detected in cells. The amino acid sequences of amino-terminal and central regions of all PP1-catalytic subunit isoenzymes are highly conserved, and the differences are mainly in the carboxy -terminal regions. Different PP1 catalytic

subunit isoforms may differentially associate with regulatory components through interactions dependent on their unique carboxy-terminal regions. The regulatory components of each PP1 isoenzyme, as yet undefined in HL-60 cells, may modulate the different translocation patterns of individual PP1 isoenzymes during monocytic differentiation[16].

The Mg^{2+}-dependent phosphatase activity of PP2C was assayed in cytosol, the nuclei and membrane fractions of HL-60 cells before and after treatment with 1,25 $(OH)_2D_3$ or all-trans retinoic acid. The exposure of exponentially growing cells to 1,25 $(OH)_2D_3$ resulted in a gradual increase in the activity of cytosolic PP2C, and the activity reached its highest level after 4 days. Concommitantly, there were significant increases in the PP2C activity in each subcellular fraction was unaltered during the course of all-trans retinoic acid-induced granulocytic differentiation. The increase in phosphatase activity of PP2C appeared to coincide with increase in the positivity of the surface marker CD11c and the extent of reduction of NBT. This data suggest that an increase in PP2C activity is associated with the 1,25 $(OH)_2D_3$-induced monocytic differentiation of HL-60. The increases in activity correlated with protein expression. The treatment of HL-60 cells with 1,25 $(OH)_2D_3$ resulted in a dramatic increase in the level of PP2Cb mRNA. The up-regulation of PP2Cb mRNA expression clearly preceded the 1,25 $(OH)_2D_3$-induced monocytic differentiation. The up-regulation of PP2C may be associated with the expression of certain differentiation markers related to monocytes, although the direct target of PP2C that is important for the HL-60 cell differentiation is unknown at present[15].

CsA and FK 506 stimulated the proliferation of HL-60 cells in vitro culture systems. It is tempting to speculate that CsA and FK506 block a process that normally exerts an inhibitory action on the event following activation and leading to DNA synthesis and cell division, whereby HL-60 cells will proliferate in a noninhibited manner. We found that immunoreactive CNA and CNB proteins, as well as Ca^{2+}-dependent calmodulin stimulated phosphatase activity, were expressed in HL-60 cells. Calcineurin activity was potently inhibited when HL-60 cells were treated with CsA or FK506. These concentrations fall within the range of therapeutic plasma levels of these drugs, and are similar to concentrations required to block T-cell activation, with FK 506 being more potent. Our data suggests that the positive effect of CsA and FK 506 on HL-60 cell proliferation is caused by the inhibition of calcineurin. Terminal differentiation is usually accompanied by growth cessation and the arrest of the differentiated cells in the G1 phase of the cycle. The treatment of cells with 1,25 $(OH)_2D_3$ led to increases in calcineurin activity in cytosol, nuclear, and membrane fractions. The increase in calcineurin activity was concordant with the increased expression of CNAa, CNAb isoenzymes, and CNB proteins that were preceded by a coordinated increase in the level of CNAa, CNAb, and CNB RNA transcripts. The transcriptional activation of CNA and CNB gene by 1,25 $(OH)_2D_3$ may, therefore, constitute a significant mechanism for regulating the Ca^{2+}-dependent calmodulin-stimulated phosphatase activity of calcineurin, independent of intracellular Ca^{2+} elevation. This mechanism may act in mediating the long-term cellular effects of calcineurin, such as cell differentiation. We suggest that the elevated expression of calcineurin is involved in the 1,25 $(OH)_2D_3$-induced inhibition of HL-60 cell proliferation, and therefore contributes to the establishment of the differentiated phenotype of monocytes. Calcineurin phosphatase activity was also upregulated during retinoic acid-induced granulocytic differentiation of HL 60 cells, and both CsA and FK506 reduced the retinoic acid-induced inhibition of proliferation. Calcineurin is primarily an inhibitory component of signal transduction in myeloid cell proliferation, and the regulated expression of calcineurin may be of importance during the normal maturation of hematopoietic precursor cells into mature myeloid cells[14].

Granulocytic Differentiation and Protein Phosphatases

ATRA induced granulocytic differentiation in cultured leukemic HL 60 cells and is clinically effective as a differentiation therapy in inducing high remission rates in patients with acute promyelocytic leukemia. The biological effects of ATRA appear to be mediated through a number of closely related nuclear retionic acid binding domains. Although the exact mechanism of the ATRA-induced granulocytic differentiation remains a mystery protein phosphorylation/dephosphorylation is also thought to be a regulatory device eminently suited for the control of the differentiation processes. Our re-

sults suggest that the PP1 enzyme is not altered during ATRA-induced granulocytic differentiation of HL 60 cells, while the PP2A activity is down-regulated during ATRA-induced differentiation. Subunit composition and specific complex formation play important roles in regulating the activity and specificity of PP2A-C. We found that the expression of PP2A-C was markedly decreased during the course of granulocytic differentiation, whereas the levels of A and Ba regulatory subunits were relatively constant. The selective down-regulation of PP2A-C may change the ratio of dimeric and trimeric PP2A holoenzyme and, therefore, may alter specific catalytic properties such as substrate specificities or the response to effectors. Treatment with ATRA led to a dramatic reduction in mRNA expression of PP2A-Cb. The down regulation of PP2A-Cb RNA transcript clearly preceded the ATRA-induced granulocytic differentiation. The mRNA level of the Aa regulatory subunit was also decreased following treatment of ATRA, while the Ba regulatory subunit remained constant. Our data suggests that the mRNA expression of PP2A-C and Aa subunits is coordinately regulated in HL-60 cells following treatment with ATRA. In spite of the early decrease in the mRNA expression of Aa regulatory subunit, the level of expression of the A subunit protein did not fluctuate throughout ATRA-induced differentiation. Probably, the A regulatory subunit is resistant to proteolysis and, therefore, has a long life span in the cell. The ATRA-induced granulocytic differentiation of HL-60 cells appears to be mediated through the nuclear retinoic acid receptor. The retinoic acid receptor is a member of the thyroid hormone superfamily of transcription factors and possesses discrete retinoic acid-binding (ligand-binding) and DNA-binding domains that regulate transcription of certain target genes. ATRA can influence PP2A gene expression either directly, by activating transcription of the PP2A gene, or indirectly, by altering the expression of the genes encoding transcriptions factors [17-19].

Methylprednisolone-induced Differentiation and Protein Phosphatases

The inhibitors of PP1 and PP2A (Okadaic Acid and Calyculin A) exhibited enhanced differentiation inducing effects on HL-60 and K 562 cells during granulocytic and monocytic differentiation[10]. This differentiation process was coupled to a PP2A regulatory subunit upregulation and an increase in the PP2A phosphatase activity in the cytosolic fractions of both cell lines (submitted paper).

Arsenic trioxide-induced Differentiation and Protein Phoshatases

The experiments done by utilizing PP1 and PP2A inhibitors (Okadaic acid and Calyculin A) and PP2B inhibitor (Cyclosporin A) demonstrated that the aforementioned phosphatases do not play any role in the arsenic trioxide induced differentiation (unpublished data).

Conclusion

In addition to the modulation of protein phosphatases, the levels of both protein kinase C and the expression of its isoforms (a,b and g) of protein kinase C was shown to increase during granulocytic differentiation of HL-60 cells induced by ATRA and dimethyl sulfoxide, 1,25 $(OH)_2D_3$-induced monocytic differentiation of HL-60 cells is also associated with an increase in both the activity of the protein kinase C and the expression of protein kinase C-a and C-b, as well as steady-state levels of mRNA of protein kinase C-a and C-b. The activation of cAMP-dependent protein kinase also appears to be involved in the regulation of HL-60 cell differentiation by TPA (phorbol ester derivative), ATRA or dimethyl formamide. Although the monocytic and granulocytic inducers share protein kinases as target molecules, it is also true that activation and/or modulation of protein kinases is solely insufficient to explain the mechanisms for bringing about terminal differentiation into granulocytic or monocytic phenotypes. Thus, the modulation of protein phosphatases plays an important role in regulating the net phosphorilation of critical substrates, that subsequently mediate the differentiation of HL 60 cells into either phenotype. PP1, PP2A, PP2B, and PP2C are thought to regulate multiple functions in vivo, including several metabolic pathways, protein synthesis, DNA replication, and the cell cycle. Differentiation of HL-60 cells by chemical agents, such as ATRA, 1,25 $(OH)_2D_3$, methylprednisolone, arsenic trioxide, and phorbol diester is accompanied by a withrawal from the cell cycle. All of these factors suggest that PP2A down regulation in ATRA-induced granulocytic differentiation, PP1 translocation, and PP2B and PP2C upregulation in monocytic differentiation are important steps in the signal transduction pathways leading to differentiation.

Acknowledgement: We are thankful to Dr. Güray Saydam in the preparation of this manuscript. Also many thanks to our colleagues at Ege Univ., Dept. of Hematology and Mie Univ., Dept of Hematology.

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