

Discussion

Most differentiated cells in adult tissues are composed of a number of distinct, irreversibly determined cell lineages, and they will maintain their specialized character. Although states of differentiation are generally stable and not interconvertible, even highly specialized cells can alter their properties to a limited extent in response to environmental cues. These facts have been notably studied using the system of differentiation of cancer cells into normal cells in the treatment with various agents, on the other hand, in plants, redifferentiation of callus cells into shoot or root in the treatment with plant hormones. I have noted a similarity of the characteristics of cancer cells and callus cells which proliferate immortally but can be redifferentiated.

I examined the effect of adenine analogs including cytokinins on the growth and differentiation of human myeloid leukemia cells, and found that some of these analogs have activities to inhibit growth and induce differentiation of human leukemia cells. Among the adenine analogs tested, cytokinins which have strong activities in plants are the most potent in inducing the differentiation of HL-60 cells, suggesting that there are some common signal transduction pathways between the differentiation of human myeloid leukemia cells and the action of plant redifferentiation-inducing hormone. Adenine derivatives have various biological and biochemical functions. 1-Methyladenine is a potent inducer of oocyte maturation in star fish (52), but it is ineffective in inducing the differentiation of human myeloid leukemia cells. Cyclin-dependent kinases (CDKs) are conserved regulators of the eukaryotic cell cycle, with different isoforms controlling specific phases of the cell cycle. Some adenine derivatives are known to inhibit CDKs. Olomoucine specifically inhibits CDC2, CDK2, and MAPK, and IPA is a relatively nonspecific protein kinase inhibitor (100,

113). IPA, but not olomoucine, is potent in inducing the differentiation of myeloid leukemia cells. Several inhibitors of protein kinase can induce the differentiation of leukemia cells (122). These results suggest that the inhibition of protein kinase(s) other than CDC2, CDK2 and MAPK may be involved in inducing the differentiation of myeloid leukemia cells. However, the significance of kinase inhibition on plant redifferentiation is not yet understood.

With respect to other plant hormones, jasmonic acid also had strong, and brassinosteroid had weak differentiation-inducing activity, but abscisic acid and auxin did not (data not shown). Cotylenin A, which has been isolated as a plant growth regulator from the metabolites of a simple eukaryote *Cladosporium* sp. exhibits cytokinin-like activity (99). Although it is structurally different from cytokinins, it also induces the differentiation of human myeloid leukemia cells (2, 123). These results suggest that there is an association between the action of plant redifferentiation-inducing hormones and the mechanisms of differentiation of human leukemia cells, although there may be not the same signal transduction pathways between them.

In part II, I examined the effect of cytokinin ribosides such as kinetin riboside, IPAR and BAR on the growth and differentiation of leukemia cells. The growth inhibition of human leukemia HL-60 cells by these adenine analogs was in the order BAR > kinetin riboside > IPAR >> IPA > kinetin > BA (Table II). Moreover, 6-methyladenine was more potent than 3-methyladenine or 1-methyladenine in inhibiting cell growth and inducing NBT reduction (Table I and Fig. I-1). These results indicate that the growth-inhibitory activity is enhanced by the transfer of relatively long side-chains such as isopentenyl, benzyl, and furfuryl residues at the N⁶-position of adenine, and greatly enhanced by the addition of ribose or deoxyribose. These modified Ado and dAdo analogs are potent inducers of apoptosis, although Ado and dAdo themselves are

not. Cytokinins, such as IPA, kinetin, and BA, are potent inducers of differentiation, however, their nucleosides did not effectively induce differentiation. Morphological and biochemical examinations revealed that cytokinins induced granulocytic differentiation and cytokinin ribosides induced apoptosis in HL-60 cells. In plants, cytokinin ribosides have almost the same biological effects as cytokinins. Although the effects of cytokinins on HL-60 cells are clearly different from those of their ribosides, cytokinin ribosides induce granulocytic differentiation in HL-60 cells in the presence of antioxidant, O_2^- scavenger or caspase inhibitor. Similar results were obtained when other human leukemia cells were treated with cytokinins or cytokinin ribosides, indicating that these different effects are not restricted to HL-60 cells. Interestingly, cytokinins have been shown to act directly as antioxidants or indirectly as regulators of antioxidants (5, 29, 46 and 103). It suggests that cytokinins may prevent accumulation of ROS which has been suggested to be a primary regulatory component followed by the activation of caspases and caspase-dependent loss of the mitochondrial membrane potential (62) in leukemia cells, too. These properties help explain why cytokinins have differentiation-inducing activity without apoptosis. In fact, cytokinin ribosides have the same differentiation-inducing activity as cytokinins in human leukemia cells, as in the case of plants, and that cytokinin ribosides induce mitochondrial disruption whereas cytokinins protect against mitochondrial disruption and apoptosis in leukemia cells.

In Part III, I examined the metabolism of cytokinins in human leukemia cells. In plants, cytokinin-binding proteins and cytokinin receptors have been isolated and shown to be involved in the cytokinin-signaling pathway (9, 47). However, this signaling pathway seems to be unlikely in human leukemia cells. The intracellular uptake of cytokinin was greatly enhanced by Ado and this increased uptake is closely correlated with the induction of cytokinin responses

in leukemia cells. The growth inhibition and differentiation of cells induced by cytokinins may require nucleotide synthesis of cytokinins. 5'-Amino-dAdo inhibits the cytokinin-induced differentiation of leukemia cells and also inhibits the uptake and conversion of cytokinin to nucleotides. These results suggest that cytokinin action in human leukemia cells is closely associated with nucleotide synthesis and is not the same as that in plant cells. In addition, Ado receptor inhibitors hardly affected the actions of cytokinins and cytokinin ribosides on HL-60 cells. These findings suggest that the mechanism of action of cytokinins and cytokinin nucleosides is closely related to adenosine metabolism that requires phosphorylation by adenosine kinase. The causal connection between Ado metabolism and the mechanism of action of cytokinin-induced differentiation remains to be elucidated.

Adenine and purine derivatives are transported across the plasma membrane by high-affinity transporters (30). This uptake is energy-dependent and occurs against a concentration gradient. When leukemia cells were treated with Ado, their ATP content increased about two-fold within 3 hours and then reached a maximal level (data not shown). However, the intracellular uptake of cytokinin and the cytokinin-induced differentiation of HL-60 cells were not significantly affected by glucose (data not shown), suggesting that an increased ATP level does not simply activate purine transporters and increase the intracellular uptake of cytokinins. Although Ado greatly enhanced the intracellular uptake of benzyladenine, Ado did not essentially affect the ratio of benzyladenine to its metabolites (nucleotides, RNA and DNA+protein), suggesting that Ado did not qualitatively affect the metabolism of benzyladenine in HL-60 cells.

Extracellular ATP and other nucleotides have been identified as important signaling molecules that mediate diverse biological effects via cell surface receptors called purinergic receptors, or P2 receptors. The

differentiation-inducing activity of cytokinins is closely associated with the synthesis of their nucleotides in HL-60 cells, and the cells express several types of purinergic receptors. The expression of P2 receptors has been shown to be affected during the granulocytic and monocytic differentiation of HL-60 cells; some receptors showed upregulation, while others showed no change or downregulation (1, 15). Extracellular ATP suppressed the growth of HL-60 cells and induced their differentiation. ATP-induced differentiation was suggested to involve the activation of cell surface P2 receptors coupled to cAMP generation and the activation of protein kinase A (100). However, the present data indicate that cytokinins suppress the growth of HL-60 cells and induce their differentiation by a mechanism distinct from that of extracellular ATP. The differentiation-inducing activity of ATP was very modest, while cytokinins were potent at inducing the differentiation of HL-60 cells. The addition of ATP or AMP only had additive effects on cytokinin-induced differentiation, i.e., neither synergism nor interference. Cytokinins did not affect cAMP generation (data not shown), and several inhibitors of adenylyl cyclase and protein kinase A also did not essentially affect cytokinin-induced differentiation. Moreover, some P2 receptor antagonists also did not affect differentiation and BA nucleotides were not released from cells into the medium in intact form. These results suggest that cytokinin-induced differentiation is not associated with signal transduction mediated by P2 receptors.

More than 30% of [¹⁴C]-BA was incorporated into RNA, DNA and protein fractions after incubation for 24 hours. Thereafter, the incorporation into RNA increased even when the labeled compound was removed from the culture. Certain cytokinin derivatives have been shown to be incorporated into some transfer RNA (tRNA) and other RNA molecules in plants (51, 78 and 112). The modification of tRNA is common in eukaryotic and bacterial tRNAs (13,117), although the function of this modification of tRNA in mammalian

cells is unknown. HL-60 variants that are deficient in the purine salvage enzyme hypoxanthine phosphoribosyltransferase can be induced to differentiate with purine antimetabolites without metabolic activation (56). Moreover, the tRNA wobble base queuine maintains the proliferative and pluripotent potential of cells in the presence of the differentiating agent 6-thioguanine (28). However, the differentiation induced by cytokinins in parental HL-60 cells required the metabolic activation of cytokinins. The incorporation of BA was not restricted to tRNA. Moreover, the labels incorporated into RNA were adenine nucleotides, not BA nucleotides. These results suggest that the differentiation-inducing effects of cytokinins are not likely due to the modification of tRNA by cytokinin

Since labeled BA contains a radioactive carbon in the adenine structure, it is possible that the radioactive compounds expel a benzyl group and turn into adenine compounds. In HL-60 cells, the conversion of BA to adenine was minimal, indicating that the labeled compounds are fairly stable. However, the labels in RNA and DNA were adenine nucleotides, not BA nucleotides, indicating that the converted adenine nucleotides were incorporated into DNA and RNA and gradually accumulated in the nucleic acids. Methylation at adenine residues of DNA controls the timing and targeting of important biological processes such as DNA replication, methyl-directed mismatch repair, and transposition (115). In addition, adenine methylation regulates the expression of at least 20 genes known to be induced during infection by *Salmonella* (34). The suppression of gene expression in eukaryotes has been found to be related to the presence of a variety of methylated bases, especially 5-methylcytosine and N⁶-methyladenine. Treatment with BA as a hypomethylating base analog decreased the level of adenine methylation in a plant. This was accompanied by a restoration of transcriptional activity in photosynthetic genes that are usually suppressed (79). Although there has been

no report that adenine methylation is associated with the differentiation of leukemia cells, several reports have proposed that DNA methylation at cytosine residues is closely associated with the suppression of differentiation and that demethylating agents can induce the differentiation of leukemia cells (77, 80, 90). These results suggest that the manipulation of adenine methylation by cytokinins might be, at least partly, involved in the induction of differentiation of myelomonocytic leukemia cells. In the present study, however, most of the labels incorporated into DNA and RNA were adenine nucleotides, not BA nucleotides, suggesting that the modification of DNA/RNA by cytokinins is minimal in human myeloid leukemia cells, even when they are incubated with high concentrations of cytokinins. BA nucleotide monophosphate is the major product in BA-treated HL-60 cells, suggesting that adenine phosphoribosyltransferase can use BA as a substrate. However, the monophosphate might be scarcely metabolized by enzymes that catalyze the phosphorylation of AMP.

In part IV, I examined the detailed mechanisms of cytokinins to induce differentiation of leukemia cells and revealed that the mechanisms were unique. As concerned about signal transduction pathways, cytokinins activated ERK1/2 in HL-60 cells before inducing granulocytic differentiation and cell cycle arrest. ERK1/2 activation by MEK was necessary for cytokinin-induced differentiation in studies using PD98059 to block MEK phosphorylation. This motivates the question of whether IPA also activated RAF (mitogen activated protein kinase kinase) as part of a typical RAF/MEK/ERK1/2 cascades. Although, the activation of ERK1/2 by IPA is extremely strong than that of ATRA or other typical inducers, IPA showed only slight increase of phosphorylation of RAF as same level as that by ATRA. These results suggest that MEK is activated through the other signal transduction systems by IPA. It have also been reported that signaling through the Ras-ERK pathway can be affected by p21-

activated kinase (PAK), an effector of the Rho family GTPase Rac and cdc42, but IPA did not increase neither Rac nor PAK. Cytokinin did not induce activation of the other member of MAPK family such as p38 and JNK/SAPK. Several inhibitors against the other signal transduction pathways did not affect the IPA-induced differentiation. These results suggest that cytokinins induce differentiation of leukemia cells primarily through activation of ERK1/2, although I cannot eliminate the possible involvement of other signal transduction pathways. Further experimentation is necessary to understand how MEK/ERK1/2 cascade is activated by IPA and the significance of ERK1/2 activation in the cytokinin-induced differentiation of leukemia cells.

Next, I examined the effect of IPA on the expression of several transcription factors that are associated with differentiation into granulocytes and monocytes, and showed that the expressions of C/EBP family proteins were differently regulated by IPA. Among the C/EBP family, C/EBP α has been reported that is required for differentiation of myeloid progenitors to granulocytes, and C/EBP β is required for monocytic differentiation of myeloid progenitor. In contrast, the functions of C/EBP δ have never been cleared well. However, the results described above suggest that C/EBP δ was important to induce differentiation of leukemia cells toward granulocyte treated by cytokinins. Recently, the in vivo profile of transcription factors during neutrophil differentiation in human bone marrow cells has been published and shows that the level of C/EBP δ dramatically increases at the stage where proliferation cease in neutrophils (7). They suggest that the differentiation pathway induced by cytokinin is closely to that of neutrophil precursors in normal human bone marrow. On the other hand, C/EBP δ also have function to regulate G₀ growth arrest and apoptosis of mouse mammary epithelial cells (45). The finding that the growth-inhibitory effect of cytokinins is more potent than that of RA or VD₃

may help to explain why C/EBP δ , not C/EBP α is implicated in cytokinins-induced differentiation.

As the knowledge about the mechanisms of differentiation by cytokinins was still limited, I used a cDNA microarray analysis to search for the cytokinin-induced genes expression in HL-60 cells. The most up-regulated gene by IPA was S100P, a member of calcium binding protein family. I investigated the expressions of S100 family including S100P, S100A8, S100A9 and S100A12 by RT-PCR, and showed that S100P was the only family member which was specifically regulated by IPA (Fig.IV-5). Although the function of S100P has been hardly elucidated so far, I showed that S100P was not accidentally increased but have important role in differentiation of leukemia cells. Treatment with MEK inhibitor PD98059 inhibited the differentiation of leukemia cells and expression of S100P induced by IPA suggest that S100P was located downstream of ERK1/2 and associated with differentiation of leukemia cells by cytokinins (Fig.IV-7). Interestingly, among the compounds I tested, only cytokinin, MJ and cotylenin A significantly up-regulated the expression of S100P mRNA and C/EBP δ (Fig.IV-6 and data not shown). These results suggest that there may be some common signals to induce differentiation of leukemia by plant growth regulators. On the other hand, S100A8 and S100A9 seem to be involved in the molecular processes leading to adhesion and/or migration, and have been associated with various inflammatory diseases (23, 24). These facts lead to the assumption that the function of S100P might be associated with not only induction of differentiation but also inflammation response, adhesion and migration independent of S100A8 and S100A9 or in a coordinated manner.

The unique and specific mechanisms of differentiation of leukemia by cytokinins led to new discovery that S100P and C/EBP δ are involved in the

differentiation of leukemia cells. This means that there are another transcriptional control and gene expression which have been unknown heretofore in the process of differentiation of leukemia cells, and the differentiation pathways are more complex and diverse than they have been considered before. Cytokinins may be useful tools for further molecular analysis and may be clue to the solution to understand network of regulatory process pathways for cell differentiation. In addition to S100P and C/EBP δ , I found that MAPK and some differentiation-associated transcription factors were regulated during induction of differentiation by cytokinins. However, the relationship of each key molecule has not been cleared, and the exact mechanism of cytokinin in inducing the differentiation of leukemia remains unknown. Further analysis will be needed.

Clinically, in a case that there is no therapeutic value in treatment with a standard protocol of anticancer drugs, the present results suggest a possibility to develop an effective way of therapy by treating inducers with specific mechanisms of differentiation such as cytokinins, by itself or with combination of others. As shown in Fig. I -5, the commitment of differentiation into granulocytes induced by IPA is early and irreversible. Cytokinin might be clinically useful because the effect of pulse treatment persists in longer resulting in less damage for patients.