

## Introduction

Plants perceive and respond to a profusion of endogenous signals such as plant hormones, and environmental cues to ensure optimal growth and development. Plant hormones mean low molecular weight compounds which regulate the physiological functions of plants including cellular growth and enlargement, differentiation of specialized tissues, and the induction of protein synthesis, and they act at a low concentration. It is also called “plant growth substance” or “plant growth regulator”. They can be divided into five principal classes: auxins, cytokinins, gibberellins, abscisic acid, and ethylene. Recently, jasmonic acid, brassinosteroids, salicylic acid, and polyamines have also been shown to have a regulatory role in plant development. One of the most crucial plant hormones, cytokinin was discovered in the search for factors that promoted division of plant cells in culture. Suggestions that plants contain chemical factors that are capable of greatly promoting cell division were made as long as 1892 (119), and in 1956, Miller et al. isolated a compound from autoclaved herring sperm DNA that had potent cell-division-promoting activity and was shown to be 6-furfurylamino-purine (69). It was given the name kinetin because of its ability to induce cell division, provided that auxin, another plant hormone was present in the medium. Following the discovery of kinetin, large numbers of N<sup>6</sup>-substituted adenine derivatives including isopentenyladenine (IPA) and benzyladenine (BA) were synthesized (59, 60). Furthermore, several naturally occurring cytokinins such as zeatin were isolated (61). They have been given a generic name “cytokinin”, because of the function which promote cytokinesis (= cell division) in the presence of auxin (40). Now, it has been known that cytokinins influence numerous aspects of plant development and physiology,

including leaf expansion, seed germination, de-etiolation, chloroplast differentiation, apical dominance, plant-pathogen interactions, flower and fruit development, and delay of senescence (74). One of the most typical physiological functions of cytokinins is the differentiation-inducing activity of adventitious bud from callus (111). Callus are clusters of dedifferentiated plant cells that are immortal and proliferate indefinitely in a disorganized manner, like human cancer cells (26). It is induced from leaf or stem in the medium containing cytokinin and auxin in equal concentrations (109). The classical reports revealed that undifferentiated callus cultures would form into roots or shoots depending on the relative amount of cytokinins and auxin in the medium; the ratio is critical rather than the absolute amount of these two hormones. When the ratio between these two plant hormones changes, callus cells acquire the competence to regenerate organs. Typically, a high auxin-to-cytokinin ratio promotes the formation of roots, whereas a low auxin-to-cytokinin ratio promotes the formation of shoots, results in the regeneration of whole plants (106, 109).

As describe above, in plants, it is simple to regulate the differentiation by changing the balance of plant hormone due to the totipotency of plant cells. On the other hand, these phenomena that dedifferentiated cells can be redifferentiated in some circumstances are known not only in plants but also in some animal cells, including cancer cells. Formation of cancer cells is based on the deregulation of normal cell growth and differentiation. This deregulation of cancer cells are caused by constitutive production of positive growth regulators, loss of the response to negative growth regulators, altered response to factors that regulate differentiation, activation of specific oncogenes or loss of suppressor genes, and it could lead to uncontrolled cell growth and a reduced probability of cellular differentiation. Although, the basic defect in cancers involves an imbalance in the relationship between the

proliferation of precursor cells and the differentiation of these cells, the occasional occurrence of spontaneous differentiation in tumors suggests the possibility that correcting dysregulatory influences may restore tumor cells to normal differentiation pathways. Indeed, many studies have demonstrated that cancer cells can be induced to differentiate under some circumstances. It was published the first reports showing that the implantation of carcinoma cells into the inner cell mass of blastocysts resulted in suppression of the transformed phenotype since such carcinoma cells participated in normal embryogenesis/development (10, 70). These observations have been substantiated by additional studies which have shown that implantation of myelogenous leukemia cells into mouse embryo suppresses transformation and allows the leukemic cells to participate in normal neonatal myeloid development (31). Related studies on neuroblastoma cells have also been reported (91). More pertinent to the consideration of differentiation inducers in the treatment of human cancers is the observation that a variety of agents can induce one or more types of transformed cells to express the characteristics of a differentiated state and cease proliferating. There are some similarities of biological phenotypes of cancer cells and callus cells, such as immortal, proliferate indefinitely in a disorganized manner and can be induced to differentiate again.

On the other hand, both of these cells have activity of telomerase, and generate auxin. As concerned about auxin, plant cells can not grow without treatment of auxin in vitro culture system. However, callus can grow independent of auxin because they generate auxin by themselves (82). Yamaki et al. detected indole-3-acetic acid, a kind of auxin in the cancer tissues and urine of cancer patients (120), and I have also isolated indole-3-acetic acid from cultured cancer cells and in the culture medium by high-performance liquid chromatography (HPLC) (data not shown). These results

indicate that both callus and cancer cells produce auxin. Furthermore, the identification of kinetin in human urine taken from patients with lung carcinoma has been reported (4). Taken together, I hypothesized that cytokinins, which induce callus to redifferentiate adventitious buds may also affect the differentiation of human cancer cells.

To prove this hypothesis that of cytokinins may affect differentiation of animal cells, I selected human leukemia cells representatives of cancer cells as experimental models. One of the reason why leukemia cells have contributed an important model system for cell differentiation is that leukemia is a cancer of blood cells whose formations are caused by the deregulation of normal blood cell differentiation. The blood contains many types of cells with very different functions, ranging from the transport of oxygen to the production of antibodies. They all have limited life-spans and produced throughout the life of the animal. They are all generated ultimately from a common stem cell in the bone marrow. This hematopoietic ( or blood-forming) stem cell is thus pluripotent, and normally divides infrequently to generate either more pluripotent stem cells (self-renewal) or committed progenitor cells, which are irreversibly determined to produce only one or a few types of blood cells. The progenitor cells are stimulated to proliferate by specific factors but progressively lose their capacity for division and develop into terminally differentiated blood cells, which have specific function including granulocytes, macrophages, lymphocytes, erythrocytes or platelets and so on. The proportion of cells at intermediate stages of the maturation pathway is tightly regulated to assure a balanced supply of functionally competent cells. If maturation is permanently arrested at an intermediate stage of development without a concomitant inhibition of cell proliferation, a population of immature cells accumulates, and proliferates indefinitely. These are leukemic cells, and they interfere with the normal functioning of

the affected organism result in variety of severe symptoms. However, numerous reports have been shown that some differentiation-inducing agents can release the block of differentiation, restore the differentiation signals, and lead to blood cells, those are almost same as normal cells. These studies have been accomplished by the use of cultured leukemia cells. This is another reason why I chose leukemia cells as experimental models that many kinds of cultured leukemia cell lines established for analysis according to the arrest at developmental stages. For example, the human myeloid leukemia cell line HL-60, derived from the peripheral blood leukocytes of a patient with acute myeloid leukemia (AML). AML cells do not undergo the cell differentiation that normally leads to mature functional blood cells. Instead, they are arrested at immature stages of development, and genes important for myeloid differentiation are often affected by chromosomal changes in AML. The arrest of maturation in myeloid leukemia cells can sometimes be reversed and can be induced to mature toward either the granulocytic (19, 20, 39) or monocytic (72, 97, 123) phenotype when they are exposed to *all-trans* retinoic acid (ATRA) or vitamin D<sub>3</sub> (VD<sub>3</sub>), respectively.

Moreover, the differentiation therapy for leukemia has been clinically well-known. Differentiation therapy is a form of treatment that seeks to alter the balance between proliferation and differentiation in a tumor and to reset homeostasis in the tumor. Since proliferative malignant cells are killed and thus eliminated, chemotherapy also restores the normal balance between proliferation, differentiation and apoptosis in the tumor. However, it is hoped that differentiation-inducing agents will be able to restore the relationship between proliferation and differentiation without the serious adverse effects associated with chemotherapy. Many studies have demonstrated that differentiation-inducing agents are effective at inducing a wide variety of transformed cell lines to differentiate and stop growing.

Differentiation-inducing agents suppress tumor growth not by killing cells, but rather by inducing an increased commitment to differentiation in immature cells, and any actual reduction in tumor size may require a long and continuous exposure of the tumor to agents, since many solid tumor cells revert to immature growing cells when the agents are removed. On the other hand, differentiated myeloid leukemia cells do not divide when the agents are removed, indicating that the linkage between differentiation and the cessation of proliferation is closer than that in solid tumors. Therefore, hematological malignancies may be more suitable for evaluating the effectiveness of differentiation-inducing agents.

Differentiation therapy, which is associated with fewer adverse effects, has been tested as a leukemia treatment modality. Differentiation therapy has been successful for treating acute promyelocytic leukemia (APL), a particular subtype of AML (17, 43). In particular, ATRA induces complete remission in more than 90% of APL patients. However, ATRA has had limited success as a single agent in the treatment of other hematopoietic malignancies (83). The further development of differentiation therapy for leukemia requires new and potent inducers of differentiation. Until now, a variety of compounds have been investigated with regard to differentiation-inducing activity, including cancer chemotherapeutic agents, hormones, vitamins, and mediators and their synthetic analogs (36). However, very few of the inducers known to date would seem to have therapeutic value in the treatment of leukemia because of their severe adverse effects or the unusually high concentration required. To develop an adequate treatment for leukemia based on the induction of differentiation, we must identify suitable drugs that can effectively induce cell differentiation. The further development of differentiation therapy for leukemia requires new and potent inducers of differentiation. It might be worthwhile to search for non-toxic

differentiation-inducing agents that can be used in clinical trials from compounds that regulate differentiation or development in other organisms, including plants and invertebrates. DIF-1(differentiation-inducing factor-1), a morphogen in a cellular slime mold, *Dictyostelium discoideum*, induced erythroid differentiation of human and murine leukemia cells (3). Differanisole A was isolated from the conditioned medium of a soil microorganism, *Chaetomium* as an inducer of the differentiation of mouse leukemia cells (84). Cotylenin A has been isolated as a plant growth regulator and is a potent and novel inducer of the monocytic differentiation of human myeloid leukemia cells (2, 37, 38 and 123). There are some reports indicating that cytokinins regulate the function not only in plants but also in other species. Rattan and Clark (1994) observed that an addition of kinetin in the culture medium of human fibroblasts delayed the onset and decreased the extent of many of the ageing characteristics (94). It was reported that cytokinin slowed the developmental process to reach maturity and prolonged the lifespan of the fruitflies (103). In a subsequent study, they reported that the egg laying capacity of kinetin-fed fruitflies was reduced drastically as compared with those kept on a normal diet (102). Hsiao et al. (2003) showed that cytokinins have effective free radical-scavenging activity in vitro and antithrombotic activity in vivo with lower the risk in mice (*Mus musculus*) (42). These results suggest that cytokinins may be a potential therapeutic agent without having severe adverse effects.

Although cytokinins are known to have pronounced effects on plant development and there is a wealth of knowledge on cytokinins chemistry, little is known about molecular mechanism including signal transduction, biosynthesis, metabolism, distribution and perception. If studies of cytokinins make further progress in animal cells, the findings are expected to deepen basic understanding of the mechanism of cytokinin's function in plant.

In this thesis, I described the effect of cytokinins on the growth inhibition and differentiation of human myeloid leukemia cells in part I, and the role of cytokinin and cytokinin ribosides in the differentiation-apoptosis switch in HL-60 cells in part II. In part III, I investigated the intracellular uptake and metabolism of cytokinin in HL-60 cells, and found that nucleotide formation is closely associated with the mechanism of cytokinin-induced differentiation. The mechanisms of differentiation induced by cytokinins were demonstrated in part IV. The changes in signal transduction pathways, transcriptional factors and differentiation-associated gene expressions were described in comparison with those by ATRA or VD<sub>3</sub>, typical inducers of terminal differentiation of the leukemia cells.