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Global cellular regulation including cardiac function by post-translational protein arginylation

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In this issue a very significant contribution to cardiology describing critical roles of ATE1 appeared (Kurosaka et al). In view of this paper, we have been asked to contribute an article (editorial) regarding ATE1(enzyme which transfers arginine from arginyl tRNA to protein acceptors). This short article consists of three sections: 1) a historical anecdote describing how ATE1 was discovered; 2) its possible role in aging and cellular transformation; 3) most importantly, its role in the development and maintenance of cardiac activity. The last section has direct bearing to the Kurosaka et al paper.

In 1962, during the course of studying cell free protein synthesis by an *E. coli* extract at Oak Ridge National Laboratory, one of the leading Institutes for studies on protein synthesis at that time, we forgot to add ribosomes to one of the test tubes. To our great surprise, C^{14}-labeled phenylalanine and leucine were incorporated into hot trichloro acetic acid (TCA) insoluble fraction, almost to the same extent as those with ribosomes! At that time, incorporation of C^{14}-labeled phenylalanine and leucine into acid-insoluble fraction was assumed to represent *de novo* protein synthesis. Normally, one would do this kind of control without ribosomes, but we did not do this control because nobody ever imagined “protein synthesis” without ribosomes. This finding that “protein synthesis” occurs in the absence of ribosomes was therefore unbelievable. Of course, the extract (soluble fraction) itself has been subjected to conventional high speed centrifugation, which had been established to remove ribosomes.

To convince ourselves and the entire laboratory, we carried out all possible experiments to examine possible residual ribosomes that could be responsible for this unexpected observation. First, the use of Model E analytical centrifuge (now found only in museums) revealed no ribosomal band. One can see the ribosome band during centrifugation with the Model E centrifuge and was used to obtain the sedimentation coefficient. Next, we examined the extract by electron microscope to look for any ribosome-like particles. No particulate components were visible under the microscope. We then fractionated the “ribosome-free” extract on sucrose gradient centrifugation, a technique considered as one of the most advanced and fashionable, at that time. The determination of the peak of amino acids incorporating activity in the extract and amino acids incorporated by the extract turned out to be sedimenting at the position corresponding to 10S. This was far lower than 70S particles (ribosome). Therefore, this system was named, “A Soluble Amino Acid Incorporating System” [1]. We then checked if amino acids other than phenylalanine or leucine could be incorporated. At this point, we had the second surprise. Amino acids other than phenylalanine or leucine were incorporated only nominally [2, 3]. When we arrived at Oak Ridge, there were about 20 postdoctoral fellows working on protein synthesis. Most of them were studying incorporation of C^{14} phenylalanine or leucine into acid-insoluble material. We asked why these amino acids were studied, and the answer was “because you get more incorporation!”

There was another reason why everybody in the lab believed that the C^{14} phenylalanine and leucine incorporation being studied must represent “protein

synthesis". At that time, it was well accepted that the addition of RNA polymerase and DNA should increase the *in vitro* synthesis of protein. Naturally, in attempts to increase "protein synthesis", we added DNA and crude partially purified RNA polymerase. As expected, the incorporation of C^{14} phenylalanine or leucine went up significantly upon the addition of partially purified RNA polymerase. This added credibility to the belief that the incorporation of C^{14} phenylalanine or leucine, indeed, represents protein synthesis. After we were convinced that the incorporation does not represent protein synthesis, we examined the partially purified RNA polymerase (protamine precipitate) and found that this fraction had a very high activity of the amino acid incorporation into the acid-insoluble material without ribosomes. After this discovery, we determined that the incorporated C^{14} phenylalanine and leucine represent the addition to the preexisting protein and is not internally incorporated [2-4]. The proteins responsible for the transfer of leucine and/or phenylalanine were named later as leucyl or phenylalanyl tRNA protein transferase (L/F-transferase). Although the crystal structure of the enzyme has been elucidated and the function appears to give the signal for protein degradation [5], the enzyme does not appear to be essential for *E. coli* survival.

Because of this unexpected finding with the *E. coli* extract, we examined if a similar phenomena could be seen in higher organisms. As in prokaryotic L/F-transferase the ribosome-free supernatant of rat liver homogenate was prepared after 100,000xg centrifugation. To our surprise, the major radioactive amino acid to be incorporated by the extract devoid of ribosome was arginine, and no significant incorporation of other radioactive amino acids was found [6]. The enzyme responsible for this transfer of arginine from arginyl tRNA to protein fraction was termed arginyl tRNA protein transferase (ATE1). This is an enzyme identified as capable of modifying proteins at the post-translational level. It functions by covalently binding arginine to the amino-terminus of acceptor proteins with acidic amino acids residues as well as side chains on different sites [7, 8]. ATE1 activity is, of course, dependent upon the cell's ability to form arginyl-tRNA, via arginyl-tRNA synthetase. ATE1 has been isolated from yeast [9], various animal tissues and cell cultures [10-12]. It has been shown to modify *in vitro* bovine thyroglobulin, angiotensin II and beta-melanocyte-stimulating hormone [12, 13]. Additionally, ATE1 modifies some of chromosomal histones, non-histone proteins [14], and membrane proteins [15]. Those exogenously added acceptor proteins that have been modified *in vitro* by the enzyme are found to contain aspartic or glutamic acid at the amino terminus, thus conferring a degree of specificity to arginine transfer [16]. Although the precise physiological function of ATE1 was not known then, we postulated that the system is closely associated with cell regulatory and/or proliferative functions, perhaps acting as a "switching" mechanism to activate or inactivate particular cellular proteins. In support of this theory were the observations that transferase activity is significantly increased in regenerating rat liver and ascites tumor cells [17]. Normal rat kidney cells transformed by Rous sarcoma virus [18], rat embryo cells transformed by dimethyl benzanthracene (carcinogen) [19] had increased transferase activity. In contrast, transferase activity decreased upon aging of human cultured cells as shown by Kaji et al in 1980 [20]. This leads to the second section dealing with the role of ATE1 in aging.

Human fibroblast strains IMR90 and WI38 have a finite life span and provide model systems for studying cellular aging. In contrast to these normal fibroblasts, SV40-transformed cells do not exhibit aging phenomena. We have examined the population doubling time, protein concentration, ATE1 and ATE1-directed modification of chromosomal proteins in non-transformed and SV40 virus-transformed cells [20, 21]. It was found that the population doubling time and cellular protein content increase as the cells enter cellular senescence, whereas the total activity of ATE1 and the ability of chromosomal proteins to accept arginine at the amino-terminal end diminished progressively during this period. Furthermore, the level of ATE1, which is associated with cellular aging in non-transformed cells, remained fixed at the amount that existed when the cells were exposed to SV40 viruses. In other words, the relative age of the culture at which SV40 transformation occurred can be revealed by the level of ATE1, because fixation of the age-associated parameter (the level of ATE1) occurs upon transformation. The transformed cells are then refractory to any subsequent aging phenomena. At the time of this discovery, we played a game of guessing the age at which stage human fibroblast cells were transformed by SV40. We gave five different transformed human fibroblast cell lines to a graduate student and asked him to determine the age at which the transformation took place. We, of course, knew at which stage the SV40 transformation occurred for each strain. It was an amazing experience for us when our graduate student identified the cellular age at which the transformation took place by measuring the amount of the ATE1 of these transformed human fibroblasts! [20]. It will be an exciting project to elucidate the precise biological significance of the decrease of ATE1 level with aging.

This finding suggested a close relationship between ATE1 and cell doubling time. Indeed, as mentioned before, this was first observed in regenerating rat liver and ascites tumor cells [17] in which a marked increase in the enzyme activity was evident with regeneration or with tumor cells. Subsequent work has shown a consistent, but not universal, relationship between cellular proliferative activity and ATE1 activity [18, 19, 22]. Aging, which is manifested as a general decrease in functional and proliferative capacity, produced a significant decline in ATE1 activity in kidney- and liver-soluble fractions derived from rats ranging in age from 2 weeks to 30 months [21]. The qualitative effect in both tissues was similar in the substrate-saturated state although the level of ATE1 in the liver preparation was quantitatively lower than that in the kidney. Of particular interest was the magnitude of the alterations in the enzyme activity, which suggests that if ATE1 can modulate protein function, the enzyme activity once again may reflect the divisional capacity of a specific tissue [21].

In view of the relatively large amounts of ATE1 within the brain, it is likely that the enzyme plays an important role in the brain [23]. However, to our surprise, neither the acceptor proteins nor ATE1 itself are affected by the aging process [21]. The exact physiological function of ATE1 cannot be accurately determined until specific endogenous acceptor proteins have been identified. Further studies of ATE1 in the brain such as Alzheimer patients' may yield some clues to this horrible disease.

The modification of chromosomal proteins [14] by the enzyme did not correlate with the level of cytoplasmic ATE1 activity, although distinct relationships between the amount of the enzyme and the age were apparent [21]. The alterations in chromosomal protein acceptor capacity in organs other than the brain are unique to the tissue in

question, and the variation in responses to the age was probably due to the diversity of chromosomal proteins that may be altered during aging. The modification of chromosomal proteins, specifically non-histone proteins, is of particular interest since they are thought to play key roles in the expression of aging phenomena. At the least, the observed changes in chromatin acceptor capacity by aging indicate the degree of chromatin restructuring or synthesis of new proteins associated with cellular senescence.

One of the reasons why we did not pursue the L/P transferase was that they were not essential [24]. Having been brought up in the age where we are taught that anything not essential is not very important, we felt that the enzyme was not worth pursuing further. Similarly, Varshavsky's work indicating that yeast ATE1 is not essential [25] dampened our enthusiasm to work on this enzyme despite many interesting features of yeast ATE1. However, our intuition guided us to work on the various aspects of the possible role of ATE1 in higher eukaryotes, despite the fact that essentiality was not established at that time. This intuition turned out to be correct, and arginylation is now well accepted to be just as important as the phosphorylation [26, 27]. The very first work, which clearly established the essential nature of ATE1 in mammals, is the experiment where the ATE1 gene was knocked out in mice, causing lethality due to heart defects in cardiovascular development and angiogenic remodeling during embryogenesis [28]. Their studies revealed that in the knockout mice, abnormal cardiac morphogenesis occurred. The underdeveloped heart had thin myocardium with immature septa. They also observed non-separation of the aorta and pulmonary artery. This is known as persistent truncus arteriosus. In other words, the authors discovered that knocking out *Ate1* led to the defects in cardiac contractility, myofibril development, and death of the embryo. ATE1 is essential! These defects became more prominent at later stages of embryonic development. However, it is unclear whether they arise through impairments in tissue signaling and/or are secondary to the onset of the embryonic lethality leading to the heart muscle disintegration. Following this finding, Rai et al [7] investigated the role of arginylation in the development and function of cardiac myocytes and their actin-containing structures during embryogenesis. They found that alpha cardiac actin undergoes arginylation at four sites during development. These findings indicate a new function of arginylation in the regulation of the actin cytoskeleton in cardiac myocytes.

The paper by Kurosaka et al in this issue together with the paper discussed above give a quantum jump to the knock-out approach, which has been used in the studies of the physiological role of ATE1. In this paper, the authors used a unique approach of knocking out only cardiac myofibril specific *Ate1.* The result was remarkable in that these mice, termed alpha MHC-*Ate1*, survived to be born as seemingly normal mice. However, the life spans of the knockout mice are much less than those of normal mice. These mice died from complications with cardiomyopathy and thrombosis after developing cardiac contractility defects in myofibril. The fact that the lack of ATE1 makes the cells "age" in cardiac contractility and other characteristics, fits to the general theme between the level of ATE1 and age as we discussed in the preceding section. Of particular importance is that this paper clearly establishes that the heart defect caused by the lack of ATE1 is not the secondary effect of general effect on

the development of whole mouse. The data indicate that essential nature of arginylation for maintaining the heart function by regulation of the major myofibril proteins and forces. Absence of ATE1 in the heart muscle leads to progressive heart failure through cardiomyocyte-specific defects.

Of particular interest is the fact that the finding may give some clues to the age-related heart failure of humans. A large number of “death due to age” can be ascribed to age-related heart failure. The close relationship between the cellular aging and ATE1 as described above and the current finding of Kurosaka et al may strongly suggest the need for the thorough investigation on the possible relationship between ATE1 and human heart dysfunction due to senescence.

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