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生命の起原および進化学会 会則

地球上における生命の起原を科学的に解明することと，生物進化の攻究により，生命体の本質を明らかにしようとする。本学会は，関係諸分野の英知を集め，互の連繋によって新しい型の総合科学を確立・発展させることにより，上記の目的達成を期するものである。

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生物の生態形成における系統的分化

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要　旨

生物進化における系統分化にともなう形態形成のすべてが、DNAによって〝決定〟されているか、について考察を試みた。その結果として、次のような結論をえた。それは、部分的にはDNAの支配下にあるが、部分的には関係する高分子相互の物理的作用が支配している、と。

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Phylogenetic Differentiation in Morphogenesis of the Organisms

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1. Introduction - definition of species -

The term "species", which discriminates one type of organisms from another, has arisen naturally over time in human societies. Therefore, the species has not traditionally been scientifically defined. The first man who gave definitions for taxonomy was C. von Linne (1758). He separated organisms on the basis of their morphological characteristics and arranged genera and species to reflect these, for example *Homo* (genus) *sapiens* (species). This morphological basis of taxonomy is the one mainly used even now. However, there are taxonomical difficulties determining the phylogenetical connections among organisms. This is because biological evolution is a continuous line in phylogeny rather than a puncuated one.

Current taxonomy principally classifies organismic characteristics on the basis of three standards: (i) morphology,(ii) fertilizability and (iii) ecological isolation. However, the greatest weight is attached to morphology, as mentioned above. Therefore, morphological species are also referred to as Linne's species.

2. Significance of morphological science

The starting point of morphogenesis in multicellular organisms is a fertilized egg. Howevre, the repeated cell division and cell death programmed during the embryogenesis establish a species-specific morphology. What is the process of embryogenesis ? Nobody can explain it yet at the molecular level, but it can be followed at the cellular or multi-

cellular level under the microscope. Embryologists have been trying to determine the course of changes in the molecular organization during embryogenesis but with little success as yet.

Recently, for example, B. Goodwin (1994) asked whether all the molecular steps of embryogenesis are determined by specific genes, and decided against it on certain grounds. He emphasizes that there are processes that are not determined by genes, base sequences of DNA, but by physical processes and properties with which the macromolecules are intrinsically endowed. Generally, it has been believed by molecular biologists that all the processes by which macromolecules are synthesized and organized in the living cell are determined by specific base sequences in DNA, and that life must be explained only by genes and their interactions. In fact, although knowledge of DNA has been rapidly accumulating, nothing has explained such life mchanisms as a whole. The reason for this is that the relation between morphogenesis and gene action has not been clarified at the molecular level. We shall look for the solution of these problems in the evolutionary theories that have been proposed in the past.

3. Evolutionary consideration of diversed biological morphologies in the biological world

(1) Lamarkian theory

J. Lamark (1801) proposed that the diverse morphologies in the nature arose when environmental actions modified the bodies of organisms. That is, the repeated actions of an environment gradually changed some related part of the body and accumulated the results. He emphasized that the modification is inheritable through generations. For example, the long neck of giraffe is explained by the fact that the animal continued to eat leaves on tall trees, and thus the neck continued to elongate. Lamark's theory says in conclusion that the environmental conditions influence body cells, and then the modified cells change genes of so-called germ cells. Finally, genetic changes occur and are inherited through generations. However, if the organ so evolved is not used for a long time, then it becomes disorganized over a number of generations and finally disappears.

According to this theory, morphogenesis of organisms evolves due to certain environmental actions. Although this theory seems easy to accept, current biology, especially molecular biology, completely denies

its verity. This is because this idea can not explain morphogenesis and evolution at a molecular level. However, it is a fact that morphology is physiologically modified. Repeated usage develops corresponding organs but the changes are never genetic. Practically, paleontology has not found continuous fossils which directly indicate evolutionary steps involved in the elongation of the giraffe's neck.

(2) Neo-darwinian theory

C. Darwin's book entitled " On the Origin of Species by Means of Natural Selection" (1859) stressed that evolved characters were acquired through natural selections repeated. In nature, individual organisms are originally different in character. Therefore, a given natural environment selects to predominate more adapted characteristics, and thus the natural conditions are the designer for morphogenesis. Neo-darwinism is a theory modified by A. Weismann (1902). According to him, evolutionary change of morphology results from "mutation on DNA and natural selection", although Darwin himself accepted Lamarkian theory in revised editions of his book. However, Weismann simply emphasized mutation and selection processes in evolution and eliminated the Lamarkian parts. Neo-darwinism is very convenient for molecular biology and is accepted by almost all biologists at present. However, some, including Goodwin (1994), have doubts about whether morphogenesis of organisms can be described so simple by a model. This problem will be discussed later.

4. Presence of homeotic genes in vertebrates and invertebrates

As all organisms have species-specific forms, it has been believed that there must be genes to determine the forms. However, these genes have not been indentified by genetic analyses of various experimental organisms. On the other hand, analysis of morphological mutants in *Drosophila* demonstrated that specific gene cluster, namely the homeotic gene box, was responsible, and further research demonstrated that such boxes are commonly contained in vertebrates and invertebrates. Therefore, the box was evolutionarily established before the phylogenetical differentiation of vertebrates and invertebrates.This must have been a very fundamental event in morphological evolution in the biological world.

Neverthless, there are tremendous differences in the forms of vertebrates and organisms more highly than coelenterates in the invertebrates. Therefore, genetic variations have occurred within the basic homeobox and accumulated over geological time. On the other hand, there is a question whether morphological variations have been dependent only on the homeobox genes. Unfortunately, current genetics has no answer for this problem. The following discussion is restricted to the significance of homeobox genes.

5. Homeobox function in tetrapod limbs and floral meristem

The concept of homeosis that was proposed by W. Bateson (1894) means formation of morphological homology such that segments repeated in bodies of vertebrates and invertebrates, such as annelids and arthropods, are homologous to each other in the structures. Recently, the homeosis has been found to be determined by a set of homeotic (Hox) genes, called the homeobox, and homeotic mutants have been isolated from not only animals but also plants. Especially, the genes controlling morphogenesis in normal embryogenesis of *Drosophila* have been deeply scrutinized. Interestingly, the homeobox has also been found in genomes of yeast and other unicellular organisms.

Tetrapod limbs are homologous among animals higher than reptiles, in which these phylogenetically originate from fins of fish. Limb bones are very durable and leave clear impressions as fossilized remains in sedimentary rocks. Therefore, they form a bridge from contemporary species back to the earlier known ancestors that existed some 400 million years ago, namely just before the stage of their emerging from the sea. The fish fins somehow underwent transformation to the more complex structures that eventually turned out to be so useful, even for playing the piano and making clay pots (Fig. 1).

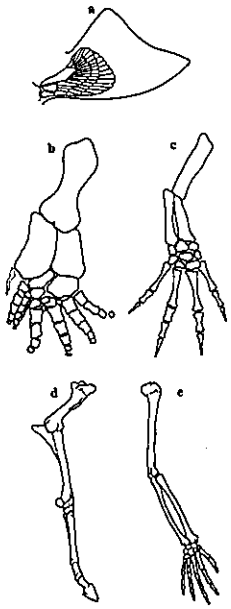


Fig. 1. Phylogenic differentiation of bone patterns of limbs in vertebrates
a: fish,
b: *Ichtyostega* (fossiled),
c: salamander,
d: horse,
e: human

It is an extraordinary thing that our limbs are essentially the same as those of all the tetrapods - horses, bats, birds, crocodiles - , and those ancients that lived in the Devonian seas. However, all these species have (or had) quite different uses for their limbs; *Ichthyostega*, fossil fish, for swimming, the salamander for swimming and walking; the chicken for flapping about; the bat for flying; the horse for walking and galloping; and humans for an endless variety of activities. As shown above, the diversity of uses indeed has led the brains of humans, and thus their cultures to more evolved levels. I will now discuss whether we could expect that only natural selection would have designed each limb to optimally serve its functions after spontaneous mutations occurred in the homeobox genes at random.

What is the basic pattern that is common to the limbs ? The answer is the following: they all begin with a single large bone, then have two more major bones, followed by a cluster of small ones that end with the terminal digits, which can vary in number between one in horse and seven in *Ichthyostega*. It is said to be eight digits in the fossilized tetrapod, *Acanthostega*.

Homeotic mutants that cause transformation of one digit into another have been found. The genes involved in these transformations are clustered together in groups on chromosomes and have common base sequences, or boxes. The Hox D (9 - 13) cluster on a chromosome of chicken hind is active in limb formation. The genes involved determine the spatial pattern of overlapping domains that provide five specific combinations of the genes and that, as a result, cause five to digits develop (Fig. 2). If one of these genes, such as Hox D11, has an extended domain of expression, covering the region where digit I arises, then this digit is transformed into a digit II - like structure - a typical homeotic transformation.

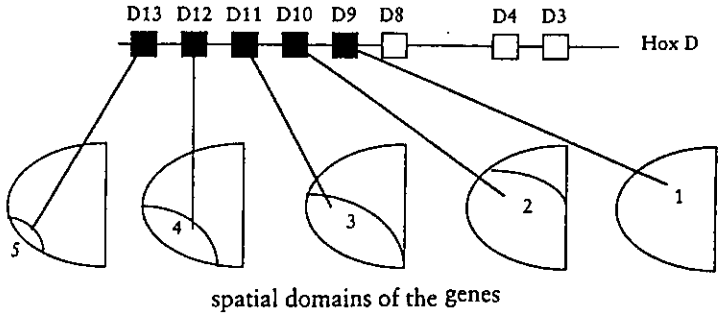


Fig.2. Relative positions of Hox D genes (homeobox) on a chicken (hind) chromosome

On the other hand, the chicken hind limb has only four digits, even though there are five combinations of the Hox D genes available. So, what the Hox genes are doing is to produce differences among digits whose number is determined by other processes. However, the maximum number of distinct digits specified by the five Hox D gene combinations is five.

The homeotic genes work in the same way also in the floral meristem; that is one organ is replaced by another structure that belongs to the same set of forms. Each of the four types of organ: sepal, petal, stamen, and carpel, is under the influence of three separate genes, A, B, and C, as shown in Fig. 3. These genes make concentric circles for their expressions that correspond to each of the whirls of organs in a flower. The combinational code for gene influence is A = sepal, AB = petal, BC = stamen, and C = carpel. This suggests that if none of these genes is active, then leaves are produced; that is, leaves are ground state.

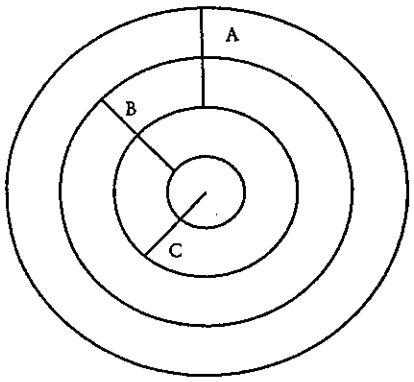


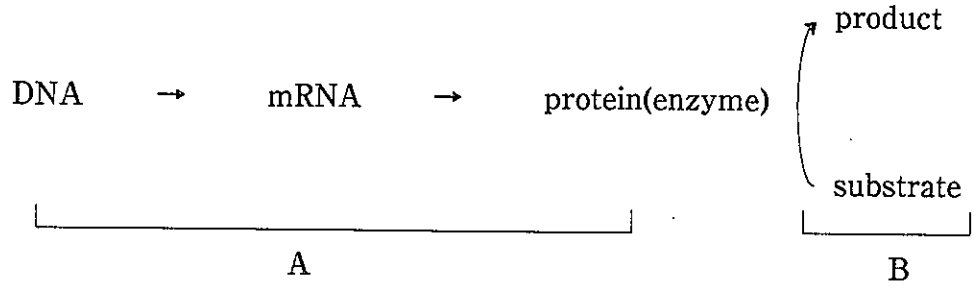
Fig. 3. The modeled patterns of homeotic genes (A, B and C)
involved in determining the structure of a colza (*Arabidopsis*)flower
A=sepals, AB=petals, BC=stamens, C=carpels

These results indicate that (i) there are homeotic genes which work for a unit of the morphology with a combination, (ii) one of these genes has an extended domain of expression and does homeotic transformation, and (iii) the morphogenesis under control of these genes is quite dynamic, depending on the environmental conditions. However, the molecular details of the morphogenetic process are unknown as yet.

6. Is it possible to reduce all morphogenesis to DNA ?

The idea is very popular even among molecular biologists that DNA determines all kinds of biological phenotypes and thus that life can be fully explained by gene actions. I'd like to reconsider as a molecular biologist whether such understanding of the life is truly reasonable.

(1) Transfer of genetic information from molecules to molecules Genetic specificity of DNA is transferred as in process A shown here.



However, the genetic information is not finally transferred in process B; the product of the enzyme reaction has no information from DNA. This is true even in syntheses of large molecules such as polysaccharides and peptidoglycans. Therefore, it does not necessarily follow that all components of the cell, as units of life, are determined by DNA. Nevertheless, such non-genetic substances, including large molecules, work to construct the living system without the information from DNA.

(2) Protein particles can automatically assemble to form active virus.

Protein and RNA subunits of tobacco mosaic virus (TMV), and of other viruses, assemble to form active *in vitro* spontaneously.

(3) Phospholipid bilayer as skeleton of the membrane.

Phospholipids are components of the membrane skeleton, and phospholipid bilayer micells or its vesicles containing internal liquid (liposomes) are automatically formed when phospholipid is suspended in water.

If the suspension was violently agitated, then the phospholipid bilayer forms vesicle, called liposome. This is just proto-cell which would be produced as the first living system in the primitive sea. The cells have integrated functional and evolved proteins and other organic substances, which have been synthesized under genetic control with their evolution.

(4) The structural genes just synthesize the informative and non-informative substances, and the regulator genes quantitatively regulate syntheses of them.

Therefore, synthesized substances form assemblages each other between same or different molecules and make cellular structures that become essential for the living organization. The morphological organization must automatically progress according to their molecular specificity. The latter specificity is determined by primary structure in the

informative molecules, such as protein, RNA, DNA, but not done in the non-informative molecules as general organic compounds. Recently, Goodwin (1994) has pointed out examples as the followings.

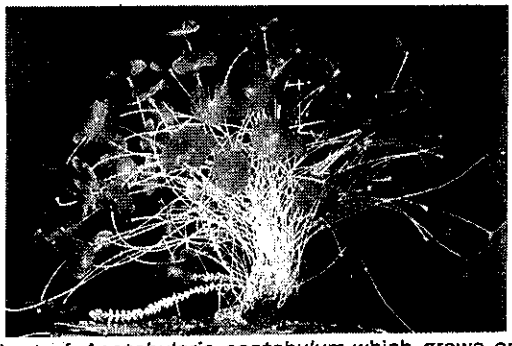


Fig. 4. A colony of *Acetabularia acetabulum* which grows on rocks of the Okinawa sea (photo. by Dr. I. Ishikawa)

(5) One of the most impressivenes for me is that an uni-cellular plant, *Acetabularia acetabulum*, growing on rocks of sea at the subtropicals can morphogenize a very complex form as shown in Fig. 4.

. However, the whorl formation of it is not so complex except final step to make the adult form after 60 days' development. A polarity of the plant is genetically determined as that (1) a rhizoid with nucleus is the center of the morphogenesis, (2) the mature whorl is formed on a tip of the stem, (3) this polarity is defined even when the stem was cut, (4) however, if the stem was cut at both the sides, then in some cases the cap grows from the both ends only once. The problem is whether such the process is genetic. Goodwin has stated that the main steps including whorl formation are to be able to simulate with a computor. Therefore, it seems to be resonable to deduce that at least the basic process of the morphogenesis is not gene-determinative but physiological or physical depending upon the accumulation and its organization of macromolecules.

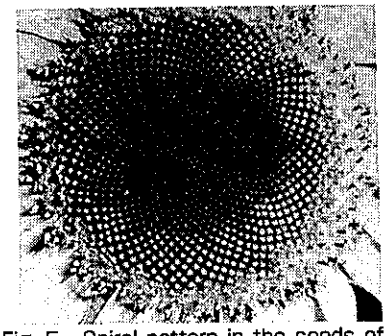


Fig. 5. Spiral pattern in the seeds of a sunflower, which is arranged according to the Golden ratio

(6) Fig. 5 shows spartial patterns in the seeds of a sun flower. The spirals series formed follow a mathematical series defined by L.Fibonacci (1828). Such phenomena also are popular in the phyllotaxy pattern of leaves on a plant. Furthermore, the similar spiral pattern can be obtained by physical experiments using oil-drops on a magnetic field.

It was explained above that the morphogenesis of tetrapod limbs, which are physical machine of the movement for vertebrates, is genetically determined by the homeobox. Many ethologists seem to evaluate this fact to show that all kinds of animal behaviors are genetic. However, understanding that very complex behaviors of the animals are down by such simple mechanisms is rash. We have to reconsider significance of the "learning", because ethology has not so long history on animal behaviors and the ethologists seems to liken to those of human.

7. Nongenetic morphogenesis

An important tenet of ethology was that animal behavior had evolved as an adaptive response to the environment. Thus, even complex behaviors could be genetically programmed to occur in response to appropriate stimuli. In that sense, C. Darwin was an early ethologist, extending his theory of natural selection beyond the shapes, sizes, and colors of animals to include their behaviors as well. However, my view is that all behaviors are on only some genetic bases, although I am not ethologist. This is because there is a tendency that takes refuge in the "gene" when the problem was scientifically difficult to explain. Anyway, we have to waite much further results from the observations and experiments on the behavior.

It has been calculated that number of nucleotide pairs per haploid genome of human is 3×10^9 (Alberts et al., 1994), and if one gene is supposed to consist of 1×10^3 nucleotide pairs,then the human genome correspods to 3×10^6 genes. Furthermore, if 1 to 2% of the total genes are functionally working as active ones, then 3×10^4 genes are practical. On the other hand, the genomes of the lower mammals can be calculated to contain 2×10^9 nucleotide pairs, namely 70% of that in human (Alberts et al., 1994). Therefore, a difference between these, 9000 genes, must function as behavioral and intelligent works of a human. In addition, the data of genome analyses show that there is a little difference in genetic constitution between human and gorilla of the primates (Patterson,1978).

These considerations suggest strongly that only minor parts, rather than all, of behaviors and intelligences of the animals would be under genetic control. However, it is a fact that behaviors that are innate are heritable and occur without any prior experience or exposure to the stimulus. Lerning is adaptive in complex, changing, and unpredictable environments, and may modify innate behavior pattern.

Basic concept to formulate the biological evolution as follows:

(i) Genes are quantitative determinants in the morphogenesis, but not qualitative ones.

(ii) The gene products assemble specifically under each of the molecular characters, and generate forms for the phylogenies that are fundamental ones such as tetrapods in the vertebrates. Therefore, the fundamental morphogenesis proceeds according to physiological snd biological principle, rather independently of the gene actions.

(iii) Many mutations in the course of biological evolution must change minor parts of the fundamental morphology and modify relatively small forms.

(iv) Therefore, even many mutations do not change the fundamental morphology, for example, invertebrates to vertebrates.

(iiv) Natural selection that is emphasized by Neo-darwinists operates on the varients which are produced after the repeated mutations. These may be illustrated as Fig. 6.

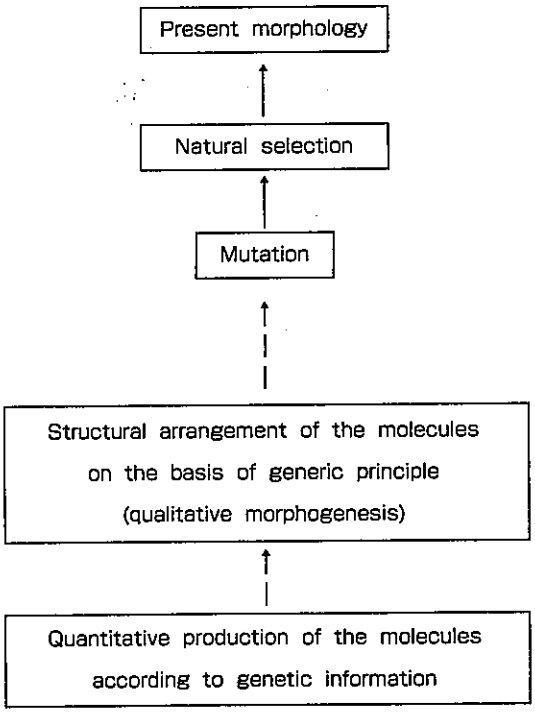


Fig. 6. Involvement of genes in the morphogenesis during morphological evolution of the organisms All the processes of the morphogenesis are determined not by the base sequences of DNA, but by the spontaneous interactions of the molecules

8. Conclusion

Neo-darwinians have believed that all of the developmental information from a fertilized egg to an adult, are stored in the genome, which is determinative source for all of the directions on the embryogenesis. However, the genes only respond to biochemical environment in the cells. The developing embryo has a field of morphogenesis, position information.

Natural selection must exclude losers from the population as a result of struggle for existence and only the victors can be predominant to occupy. If the process was repeated in the nature for four billion years, then finally the strongests had to have occupied on the earth. If it is true, humankind will correspond to the victor. (If so, they must select for the strongest race through repeated war. It is a truth of the evolution theory.) Can we humankind survive without other organisms ? It is apparent that we completely depend on foods, oxygen, and circumstance to live. Although I do not like to discuss further, we need affluent ecosystem. Furthermore, mutations that we now understand make a minor difference in character among wild types and varients. Therefore, large differences of the morphologies in systematics as shown by family, order, class, and phylum (or division) are difficult to explain only by the mutations. Anyway, term adaptation is quite unclear in its difinition.

Referrences

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. (1994) *Molecular Biology of The Cell (3rd edition)*, Garland Publishing, Inc., New York.

Goowin, B. (1994) *How the Leopard Changed Its Spots - The Evolution of Complexity* -, Charles Scribner's Sons, New York.

Patterson, C. (1978) *Evolution*, The British Museum, London.

Bateson, W., Darwin, C., Fibonacci, L., Lamark, J., von Linne, C. and Weisman, A. are classic scientists. Therefore, their literatures cited are not written as the references.

Study of the relation between the variation of HIV and the disease progression by entropy evolution rate

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ABSTRACT

In order to measure the difference of genetic sequences some quantities introduced from information theory are useful. In this study, we analyzed the relation between the variation of HIV and the clinical course of HIV infected patients by means of an information measure, called the entropy evolution rate. The region used in our analysis is the V3 region, which was successively obtained from patient at every stage. From these results, we can infer the condition of the disease from the variation pattern of the entropy evolution rate.

Key word: entropy evolution rate, HIV, V3 region

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エントロピー進化率によるHIV変異と症状変化の関連性についての検討

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1．はじめに

AIDSの原因ウイルスであるHIVは，その突然変異率は非常に高く，なかでもHIVの外被糖タンパクgp120とgp41をコードしているenv領域はHIVの遺伝子配列の中でも，特に変異率が高いことが知られている．また，外被糖タンパクgp120には可変領域（V1～V5）が存在するが，このうち，3番目に位置するV3領域はgp120のアミノ酸数296番目と330番目の2つのシステイン残基に囲まれた部分で，35個のアミノ酸から構成されいる[1,2]．また，このV3領域はループ構造をしているのでV3ループとも呼ばれている．このV3領域はHIVの中和抗体エピトープと細胞傷害性T細胞の認識エピトープが存在することから，免疫学的に大変重要であることが知られている．さらに，この領域にはSI(Syncytium-Inducing)型，マクロファージ親和性，T細胞親和性といったウイルスの性状を決めるアミノ酸サイトが存在する事が分かっている[3,4,5,6,7,8]．

我々はHIVに感染した患者から採取された遺伝子配列を，系統的な面からと情報論的な面から捉えることにより，患者の病気の進行過程が予想できると考えられる．本論文では，情報理論的な面から考察する．すなわち，情報理論の基礎概念であるエントロピーや相互エントロピーの概念を基に導入された遺伝学的差異（エントロピー進化率(EER)[9]）を用いて，同一患者から時間を追って，採取さ

れた HIV の遺伝子配列の変異過程を調べる．文献[10]では HIV 変異過程を各患者毎に調べ，エントロピー進化率による HIV 変異の平均値は AIDS 発症時にはピークをとることが予測されている．

そこで本論文では，先に述べた V3 領域の変異を見ることの重要性と V3 領域に注目し，かつ，個々の患者だけでなく，全患者のデータを総合して病状の変化過程をエントロピー進化率を用いて，解析を行う．つまり，V3 領域の変異過程と患者の症状の変化との関連性を調べ，また，エントロピー進化率による HIV 感染－AIDS 発症－死亡における V3 領域の変異過程を総合的に考察する．

2. エントロピー進化率

ここでは，解析で用いるエントロピー進化率[9]について説明する．まず，アライメントされた 2 つの生物種のアミノ酸配列$\mathcal{A},\mathcal{B}$に対して，完全事象系を定義すると，

$$\begin{pmatrix}\mathcal{A}\\ p\end{pmatrix}=\begin{pmatrix}* & A & C & \cdots & W & Y\\ p_0 & p_1 & p_2 & \cdots & p_{19} & p_{20}\end{pmatrix}$$

$$\begin{pmatrix}\mathcal{B}\\ q\end{pmatrix}=\begin{pmatrix}* & A & C & \cdots & W & Y\\ q_0 & q_1 & q_2 & \cdots & q_{19} & q_{20}\end{pmatrix}$$

となる．ここで，p_i, q_jはそれぞれアミノ酸配列$\mathcal{A},\mathcal{B}$における＊及びアミノ酸（20 種）の出現確率である．また，2 つのアミノ酸配列$\mathcal{A},\mathcal{B}$に対して完全事象系$(\mathcal{A},p)$，$(\mathcal{B},q)$が与えられたとき，完全複合事象系は

$$\begin{pmatrix}\mathcal{A}\times\mathcal{B}\\ r\end{pmatrix}=\begin{pmatrix}** & *A & \cdots & YY\\ r_{00} & r_{01} & \cdots & r_{2020}\end{pmatrix}$$

となる．ここで，r_{ij}はアミノ酸配列$\mathcal{A},\mathcal{B}$において，アミノ酸及び＊が同時に出現する同時確率分布である．

各生体の遺伝子列に対する完全事象系，完全複合事象系が設定されるとアミノ酸配列（塩基配列）の各種エントロピーが以下のように定義できる．

$$S(\mathcal{A})=-\sum_i p_i \log p_i$$

$$I(\mathcal{A},\mathcal{B})=\sum_i\sum_j r_{ij}\log\frac{r_{ij}}{p_i q_j}$$

ここで $i,j=0,1,\cdots,20$ である.

エントロピー$S(\mathcal{A})$は生物種$\mathcal{A}$が持つ情報量を表し，相互エントロピー$I(\mathcal{A},\mathcal{B})$は$\mathcal{A}$と$\mathcal{B}$との間での情報のやりとりの精度を表す．この2種類のエントロピーを用いて，生物間の類縁度を測るためのエントロピー比 $r(\mathcal{B}|\mathcal{A})$ が次のように定義されている.

$$r(\mathcal{B}|\mathcal{A})=I(\mathcal{A},\mathcal{B})/S(\mathcal{A})$$

この $r(\mathcal{B}|\mathcal{A})$ は$\mathcal{A}$がもつ情報量と，$\mathcal{A}$から$\mathcal{B}$へ伝達された情報量の比であり，情報論的に見ると$\mathcal{A}$に対する$\mathcal{B}$の類似度を表す尺度と考えることができる．この値は $0\le r(\mathcal{B}|\mathcal{A})\le 1$ であり$\mathcal{A}$と$\mathcal{B}$との間の類似が大きくなると$r(\mathcal{B}|\mathcal{A})$の値は大きくなる.

また，対称エントロピー比$r(\mathcal{A},\mathcal{B})$が次のように定義されている.

$$r(\mathcal{A},\mathcal{B})=\frac{1}{2}\{r(\mathcal{A}|\mathcal{B})+r(\mathcal{B}|\mathcal{A})\}$$

この対称エントロピー比$r(\mathcal{A},\mathcal{B})$をベースにして遺伝的差異$\rho(\mathcal{A},\mathcal{B})$を

$$\rho(\mathcal{A},\mathcal{B})=1-r(\mathcal{A},\mathcal{B})$$

で定め，この$\rho(\mathcal{A},\mathcal{B})$をエントロピー進化率という．また，この値は$0\le\rho(\mathcal{A},\mathcal{B})\le 1$ であり，$\rho(\mathcal{A},\mathcal{B})$の値が小さいほど2種の生物の類似度は大きいといえる.

3. 患者のデータ

我々はこの解析にあたり，文献[2,5,6,11,12,13]で報告されている16人のHIV患者から定期的に採取されたV3領域の塩基配列を集めてきた．なお，これらの配列のデータは塩基配列のデータベース（DDBJ/EMBL/GenBank）に登録されている．我々の解析では，これらの患者16人を，Patient A～Pと命名した．また，これらの患者の臨床データ等をTable 3.1に要約した.

Designation in our analyses	Patient A	Patient B	Patient C	Patient D	Patient E	Patient F
Designation in the original paper	1	495	82	s1	s2	s4
Presumed transmission mode	Homosexual contact	Homosexual contact	A single batch of factor Ⅷ	No information	No information	No information
Clinical status	Asymptomatic	AIDS in 1989	Asymptomatic	No information	No information	No information
CD4 counts	Decreasing	Decreasing	Decreasing	Fluctuating	Decreasing	Decreasing
Antiviral therapy	None	AZT from 1989	None	None	None	None
Term for the study	1985~after 59 months	1985~after 56 months	1984~1991	1985.11~1989.5	1985.5~1987.10	1985.1~1989.6
Tissue	Serum	Serum	Plasma	PBMC	PBMC	PBMC
Molecular type	Viral RNA	Viral RNA	Viral RNA	DNA	DNA	DNA

Designation in our analyses	Patient G	Patient H	Patient I	Patient J	Patient K	Patient L
Designation in the original paper	P1	P2	P3	P4	P5	P6
Presumed transmission mode	No information	No information	No information	No information	No information	No information
Clinical status	Died within 36 months	Died within 42 month	No information	No information	No information	No information
CD4 counts	Rapid Progress	Rapid Progress	Moderate Progress	Moderate Progress	Stability	Stability
Antiviral therapy	None	AZT from 30 months	AZT from 48 months	AZT from 64 months	None	None
Term for the study	1985~after 32 months	1985~after 35 months	1986~after 122 months	1986~after 116 months	1984~after 47 months	1985~after 93 months
Tissue	PBMC	PBMC	PBMC	PBMC	PBMC	PBMC
Molecular type	DNA	DNA	DNA	DNA	DNA	DNA

Designation in our analyses	Patient M	Patient N	Patient O	Patient P
Designation in the original paper	ACH0039	ACH0208	P198	6052
Presumed transmission mode	Homosexual contact	Homosexual contact	No information	No information
Clinical status	AIDS in 1990.11	AIDS in 1990.6 And 1993.9 Died	AIDS	AIDS
CD4 counts	No information	Decreasing	Decreasing	Decreasing
Antiviral therapy	None	None	None	None
Term for the study	1987.10~after 37 months	1985.12~after 93 months	52 months	47 months
Tissue	PBMC	PBMC	PBMC	PBMC
Molecular type	DNA	DNA	DNA	DNA

Table 3.1 Data used for our analysis

表からも分かるように，Patient A と Patient B は 1985 年の初期感染から約 5 年間，追跡調査されている[2]. Patient A は感染から 33 ヶ月目に p24 抗原血症が再発しているが AIDS 発症に至っていない. Patient B は CD4 値が減少し，55 ヶ月後に AIDS に発症し，AZT 治療が行われた.

なお，CD4 値とは体内の免疫細胞の数を表し，健康な人は普通 $1mm^3$ あたり 1000 個ほどであるが，HIV に感染すると徐々に減少し，$1mm^3$ あたり 100 個以下まで下がることが知られているので，進行の度合いを見る指標の 1 つとして使われている．また，CD4 値が 200 を切ると，AIDS と診断される（CDC（米国・国立防疫センター）の診断基準による）.

Patient Cは1984年に感染し，7年間調査，研究された[11]．この患者は初期感染から7年目にはCD4値が200を切っているが，AIDSに発症していない．

Patient D～F[12]については，採取された月ごとのCD4値が示されており，Patient Dは0ヶ月目が470，20ヶ月目が826，26ヶ月目が273，42ヶ月目が515と変動している．Patient Eは0ヶ月目が1225，23ヶ月目に756，29ヶ月目には368と減少している．Patient Fは0ヶ月目が943，48ヶ月目に575，53ヶ月目には187という低い値になっている．

Patient G～L[13]についてはCD4値の減少の仕方で3タイプに分けられ，G，HはCD4値が急速に減少している患者で，I，JはCD4値がなだらかに減少してしている患者で，K，LはCD4値が安定している患者である．また，Patient H，I，Jはそれぞれ初期感染より30ヶ月目，48ヶ月目，64ヶ月目からAZT療法が行われた．Patient G，Hはそれぞれ36，42ヶ月目に死亡している．

Patient M[5]は初期感染から37ヶ月目にAIDSと診断された．

Patient N[5,6]は，初期感染から5ヶ月目から79ヶ月目にわたりデータが採取された．この患者は初期感染から54ヶ月目にAIDSと診断され，93ヶ月後に死亡した．

Patient O[6]は初期感染時が不明だが，AIDSと診断される10ヶ月前からAIDS後15ヶ月目まで，データが採取された．そして，AIDS発症後から42ヶ月目に死亡した．

Patient P[6]もPatient Oと同様，初期感染時が不明だが，AIDSと診断される7ヶ月前からデータが採取され，AIDS発症後から40ヶ月目に死亡した．

いずれの遺伝子配列のデータは，HIVで特に変異率の高いと言われる外被糖タンパク質gp120コード領域の一部分であり，本稿ではこれらの配列からV3領域のみをピックアップして解析を行った．

4. エントロピー進化率によるV3領域の解析

4.1 解析方法

集めてきたV3領域の遺伝子配列のデータを次の指標で解析を行った．HIVの遺伝子配列が前回採取された月に対してどの程度変化が現れたかを比較するために，採取した月のデータと前回採取した月のデータとのエントロピー進化率を計算し，平均値を出した．

以下，具体的に例を挙げて説明してみる．

Patient Aで用いる配列のデータの数は各採取月ごとに，0ヶ月目（採取開始月）は8個で，13ヶ月後は7個，22ヶ月後は9個，33ヶ月後は9個，46ヶ月後は9個，59ヶ月後は8個である．

そして，アライメントを行った配列に対して，すべての組み合わせのエントロピー進化率を計算する．例えば，感染から 13 ヶ月後の配列A_i^{13}と 22 ヶ月後の配列A_j^{22}のエントロピー進化率$\rho\left(A_i^{13}, A_j^{22}\right)$ $(i=1,\cdots,7. j=1,\cdots 9)$は 63 通り求められる．したがって，それらの平均値は次のように与えられる．

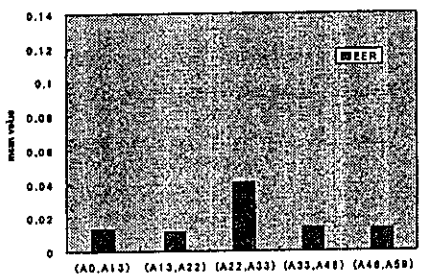
$$\overline{\rho}\left(A^{13}, A^{22}\right)=\frac{\sum_{i=1}^{7}\sum_{j=1}^{9}\rho\left(A_i^{13}, A_j^{22}\right)}{63}$$

同様な方法で，$\overline{\rho}\left(A^{0}, A^{13}\right)$，$\overline{\rho}\left(A^{22}, A^{33}\right)$，$\overline{\rho}\left(A^{33}, A^{46}\right)$，$\overline{\rho}\left(A^{46}, A^{59}\right)$を計算する．

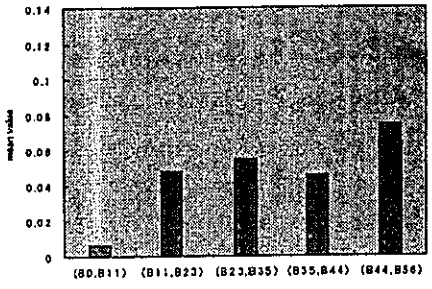
以上，残りの患者についても Patient A と同様に計算する．なお，エントロピー進化率を求めるのに際して MOU-アライメント[14]を使用した．

4.2 解析結果

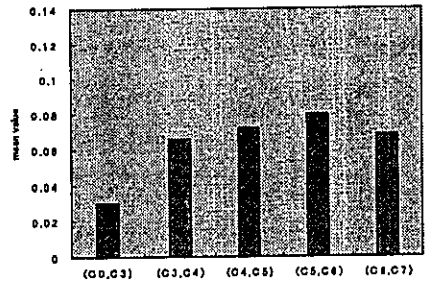
前回の調査月に対する V3 領域におけるエントロピー進化率の平均値の結果のグラフを Fig 4.1 に示す. Fig 4.1 では全 16 人より Patient A, Patient B, Patient C, Patient N, Patient P の 5 人の患者の結果を載せた．なお，(Ai,Aj)は Patient A の i ヶ月目と j ヶ月目のエントロピー進化率の平均値を表わしている．他の患者も同様であるが，Patient C では"月"ではなく，"年"を表わしている．



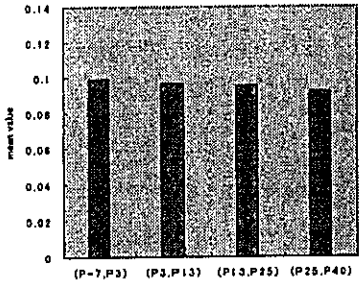
Patient A



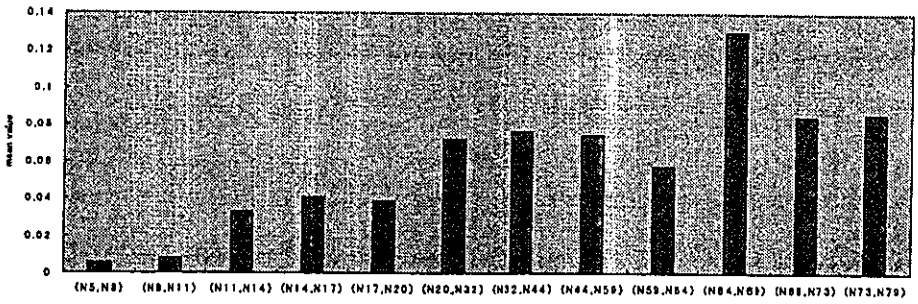
Patient B



Patient C



Patient P



Patient N

Fig 4.1 Mean of the entropy evolution rate(bars) for each stage

Fig 4.1 の各月毎のエントロピー進化率の平均値の変化を見ると，Patient A は p24 抗原血症が再発した 33 ヶ月目にエントロピー進化率の平均値の極端な増加が見られる．また，Patient B は 11 ヶ月目～23 ヶ月目にかけて，1 度目のエントロピー進化率の平均値の変化が見られ，次いで，AIDS に発症した 55 ヶ月後に極端な増加が見られる．

Patient C は 3 年目～4 年目に値の大きな増加が見られ，Patient B の変異のパターンと似ているので，AIDS 発症が近いように思われる．

Patient P のグラフの－7 ヶ月は AIDS になる 7 ヶ月前のことである．AIDS 発症後，少しずつエントロピー進化率の平均値が減少していることが分かる．Patient N は初期感染から 54 ヶ月目に AIDS と診断されているが，その後 64 ヶ月後から 69 ヶ月後にかけてエントロピー進化率の平均値が大きく変化している．

これらの結果を見ると，V3 領域の変化をエントロピー進化率から解析した場合，病気の進行過程に併せたエントロピー進化率の変化のパターンが見えてくる．次節では，これらの結果を基にエントロピー進化率による HIV 感染－AIDS 発症－死亡における V3 領域の変異過程を総合的に考察する．

5. エントロピー進化率による V3 領域の変異過程

5.1 考察方法

この節では，前節 4 で求められた結果を踏まえ，エントロピー進化率による V3 領域の変異の全過程を示す．

まず，エントロピー進化率における時間の間隔を統一するために，各患者に対して約 9～13 ヶ月程度の間隔で，エントロピー進化率を求める．どの時間を取ったかは次の Table 5.1 にあげる．この表で，太字の部分が採用した患者で，表中の数字は各患者で HIV の遺伝子配列が採取された月（Patient C は年），太字で枠内が網かけになっているのがこの論文において用いたデータの採取月である．

patient	Patient A	Patient B	Patient C	Patient D	Patient E	Patient F	Patient G	Patient H
time point	0	0	0	0	0	0	9	9
	13	11	3	23	20	48	13	9
	22	23	4	29	26	53	16	15
	33	36	5		42		22	18
	46	44	6				28	21
	59	56	7					27
								31
								38

patient	Patient I	Patient J	Patient K	Patient L	Patient M	Patient N	Patient O	Patient P
time point	7	8	3	19	10	5	7	10
	10	21	9	22	17	8	6	3
	22	25	19	29	20	11	15	18
	25	37	23	39	25	14		35
	49	49	28	43	29	17		40
	55	54	34		37	20		
			47			32		
						44		
						59		
						64		
						69		
						73		
						79		

Table 5.1 Month when sequence is chosen

Table 5.1 のようにピックアップしたデータを基にして，エントロピー進化率を求める．エントロピー進化率を求めた後，次の 2 つの場合において，エントロピー進化率による V3 領域の変異過程を考えてみる．

（I）AIDS 発症時期を基準にした場合

（II）初期感染時を基準にした場合

（I）は AIDS 発症時期を基準にしたもので，調査期間中 AIDS に発症していない患者については各患者の臨床データ，例えば，CD4 値などを参考に感染初期か中期，末期と時期を割り当てた．

（II）は初期感染時（0 ヶ月目）を基準にしたもので，AIDS 発症時には関係のないものである．

5.2 結果

5.2.1 AIDS 発病時期を基準にした V3 領域の変異過程

5.1 節より，まず（I）の場合において全過程を考察してみる．AIDS 発症時期を基準（Table 5.2 の網かけで太字の部分）に約 12 ヶ月間隔で各患者のエントロピー進化率を求める（Table 5.2 では EER の列）．そして，各行の患者のアミノ酸配列（例えば，Patient B なら AIDS 発症時期は(B48,B54)）のエントロピー進化率がその時間におけるエントロピー進化率の値とし，また各行で数人の患者が重なっている部分のエントロピー進化率の値は平均をとり，その平均値をその時間におけるエントロピー進化率の値とする．

symptom of diease	EER							
Asymptomatic		(L19,L29)						
		(L29,L42)						
	(A0,A13)							
	(A13,A22)							
	(A22,A33)			(K9,K23)				
	(A33,A46)			(K23,K34)				
	(A46,A59)	(B0,B12)	(C36,C48)	(K34,K47)				
		(B12,B24)	(C48,C60)	(J8,J21)		(N8,N20)		
ARC		(B24,B36)	(C60,C72)	(J21,J37)	(M10,M17)	(N20,N32)		
		(B36,B48)	(C72,C84)	(J37,J48)	(M17,M29)	(N32,N44)		
AIDS		(B48,B54)			(M29,M37)	(N44,N59)	(P7,P8)	(O7,O6)
						(N59,N69)	(P3,P13)	(O6,O15)
		(G9,G16)	(H3,H15)			(N69,N79)	(P13,P25)	
		(G16,G26)	(H15,H27)				(P25,P40)	
			(H27,H35)					

Table 5.2 Sequence data around the appearance of AIDS

この Table 5.2 を基にグラフにしたのが次の Fig 5.1 である.

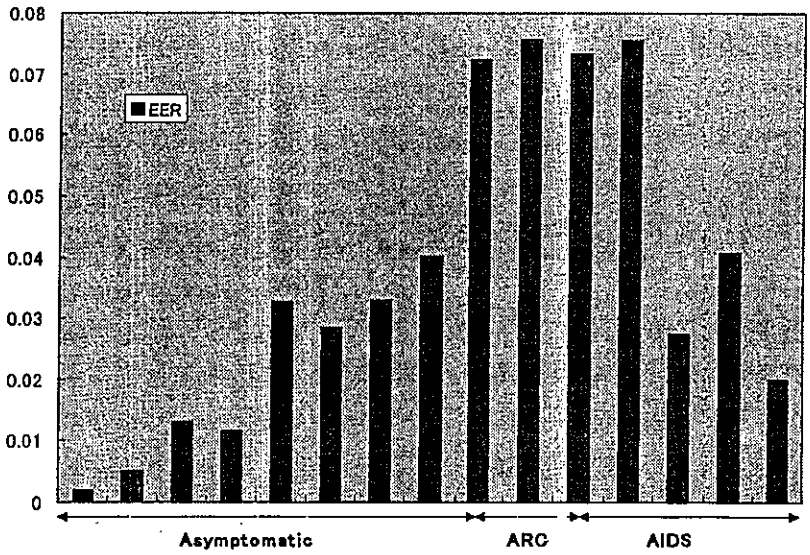


Fig 5.1 Variation process of the V3 region by EER around AIDS

5.3 調査開始月を基準にした V3 領域の変異過程

次に 5.1 節で述べた指標（II）の場合を考えてみる．初期感染時（Table 5.3 の網かけで太字の部分）を基準に約 12 ヶ月間隔で各患者のエントロピー進化率を求める（Table 5.3 では EER の列）．そして 5.2 節と同様に，各行のエントロピー進化率がその時間におけるエントロピー進化率の値とし，また各行で数人の患者が重なっている部分のエントロピー進化率の値は平均をとり，その平均値をその時間におけるエントロピー進化率の値とする.

symptom of disease	EER							
Asymptomatic	(A0,A13)	(B0,B11)	(G0,G9)	(H8,H15)	(J8,J21)	(K9,K23)	(M10,M17)	(N8,N20)
	(A13,A22)	(B11,B23)	(G9,G16)	(H15,H27)	(J21,J37)	(K23,K34)	(M17,M29)	(N20,N32)
	(A22,A33)	(B23,B35)	(G16,G26)	(H27,H35)	(J37,J48)	(K34,K47)	(M29,M37)	(N32,N44)
ARC	(A33,A46)	(B35,B44)	(C36,C48)					(N44,N59)
AIDS	(A46,A59)	(B44,B56)	(C48,C60)					(N59,N69)
			(C60,C72)					(N69,N79)
			(C72,C84)					

Table 5.3 Sequence data from primary infection

この Table 5.3 を基にグラフにしたのが次の Fig 5.2 である.

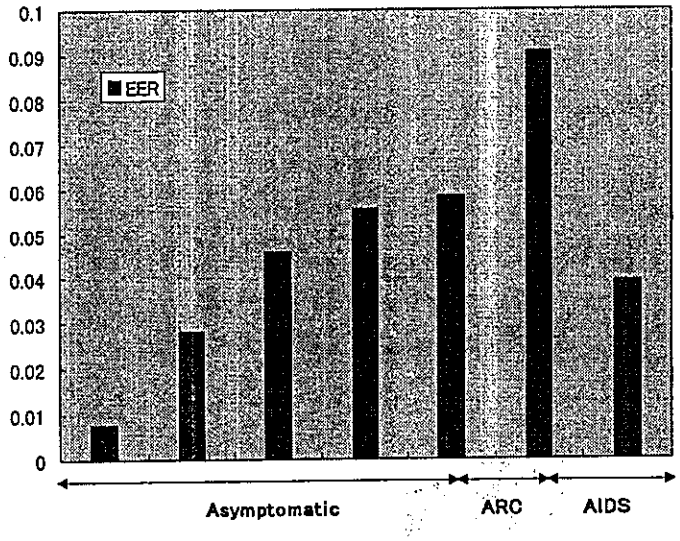


Fig 5.2 Variation process of the V3 region by EER from primary infection

5.4 各場合についての考察

次の 2 つの指標に従い，エントロピー進化率による V3 領域の変異過程を考えてみたが,

（I）AIDS 発症時期を基準にした場合

（II）初期感染時を基準にした場合

Fig 5.1, 5.2 を見ると，各場合ともに同じような結果が得られた．すなわち，初期感染時から感染中期 (急性感染, 無症候期(Asymptomatic)) , AIDS 発症時 (ARC (AIDS 関連症候群)・AIDS) , 感染末期 (AIDS) と一般的に言われている AIDS の臨床症状ごとにエントロピー進化率の値の変化が見られる.

まず，初期感染時にはエントロピー進化率の値は低い，まだ，免疫系から逃れるために必要な進化が始まり，淘汰される前であると思われる．無症候期は，臨床的には無症候であっても，ウイルス学的にはこの時期は活発に活動しているこ

とが分かっているが，我々の考察においてもそのことが反映されており，徐々に変異してることが分かる．そして，ARCに進行していくと，ウイルスは変異を繰り返し，淘汰され，病原性の強いものが血液中に出現するが，この時期のエントロピー進化率の値は非常に高くなっていて，AIDS発症前後にその値はピークを迎えている．感染末期時はエントロピー進化率の値は下がり始めているが，これは感染者の免疫能力がかなり落ちていることから，V3領域がこれ以上の進化をする必要に至っていないということと思われる．

また，各場合について我々の結果から次のことが分かる．（Ⅰ）の場合，AIDSに発症していない患者のデータに対してはAIDSに発症したと診断がされていなくても，患者の詳しい臨床データがあれば，患者がどのステージにあるか，より正確に当てはめることができると思われる．一方，（Ⅱ）の場合，各患者の調査開始月が，必ずしも初期感染とは限らないという欠点が残るし，AIDS発症時期が各患者によってばらつくため，AIDS発症時期にエントロピー進化率の値がピークを迎えることを考えると，AIDS発症時期が早い患者と遅い患者のデータの量の偏りよって，グラフの結果が左右しそうである．

6. 結論

4.2節の結果から分かるように，HIVの変異過程を見ていくとき，V3領域の遺伝子配列の変化に注目することは非常に重要であると思われる．解析結果より，エントロピー進化率によるV3領域の変化とAIDSの臨床症状との関連性があると思われ，エントロピー進化率の変化の仕方はある種のパターンを示唆するような結果が得られている．このことから，これらの結果をもとにV3領域におけるエントロピー進化率の変化を2つの場合において考えてみたが，どちらの場合もAIDSの臨床症状に沿ってエントロピー進化率の値の変化に特徴が出ている．

エントロピー進化率におけるV3領域の変異の過程は，エントロピー進化率の変化のパターンから，病気の進行過程に併せた変化のパターンが見えてくる．これを見ると，感染初期からエントロピー進化率の平均値が上昇して安定した頃，無症候期となり，その値が再び上昇した頃に，ARC期に移行し，やがてAIDSと診断される．そして，その後変化の値は減少していき，死に至ると思われる．

AIDS発症は様々な要因が重なったときに成立するものと思われる．患者の体質，免疫力などの個人的格差も影響すると考えられる．その一つの大きい要因であろうV3領域のおける役割を考えると，V3領域の解析は重要と思われ，また，この領域の変異の進行具合はAIDS発症時期の予測の上で，重要と思われる．AIDSの進行具合をV3領域の変異にのみで判断するのは難しいが，一つの判断材料として，この指標にエントロピー進化率を用いることは有用であり，こうした

解析により病気の進行過程を記述できると思われる.

文献

[1] J.D.Watson,M.Gilman,J.Witkowski,and M.Zoller; Recombinant DNA Second Edition, Scientific American Books, W.H.Freeman and Company N.Y.(1993)

[2] T.W.Wolfs,G.Zwart,M.Bakker,M.Valk,C.Kuiken,and J.Goudsmit; Naturally occurring mutations within HIV-1 V3 genomic RNA lead to antigenic variation dependent on a single amino acid substitution, Virology 185,pp.195-205(1991)

[3] J.J.De Jong,A.De Ronde,W Keulen,M Tersmette,J. Goudsmit; Minimal requirements for the HIV-1 v3 domain to support the SI phenotype : analysis by single amino acid substitution, J. Virol.66,6777-6780(1992)

[4] R.A.M.Fouchier,M.Groenink,N.A.Kotstra,M.Tersmette,H.G.Huisman,F.M-iedema,H. Schuitemaker; Phenotype-associated sequence variation in the third variable domain of the HIV-1 gp120 molecule, J.Virol.66,3183-3187(1992)

[5] A.B.van't Wout,H.Blaak,L.J.Ran,M.Brouwer,C.Kuiken,H.Schuitemaker; Evolution of Syncytium-Inducing and Non-Syncytium-Inducing Biological Virus Clones in Relation to Replication Kinetics during the Course of Human Immunodeficiency Virus Type 1 Infection, Journal of Virology,Vol.72,5099-5107(1998)

[6] A.B.van't Wout,L.J.Ran,C.L.Kuiken,N.A.Kootstra,S.T.Pals,H.Schuitemaker; Analysis of the Temporal Relationship between Human Immunodeficiency Virus Type 1 Quasispecies in Sequential Blood Samples and Various Organs Obtained at Autopsy, Journal of Virology,Vol.72,488-496(1998)

[7] T.Shioda,J.A.Levy,C.C.Mayer; Small amino acid changes in the V3 hypervariable region of gp120 can affect the T-cell-line and macrophage tropism of HIV-1, Proc.Natl.Acad.Sci.USA,Vol89,9434-9438(1992)

[8]T.Shioda,S.Oka,S.Ida,K.Nokihara,H.Toriyoshi,S.Mori,Y.Takebe,S.Kimura,K.Shimada, Y.Nagai; Naturally occurring single basic amino acid substitution in the v3 region of the HIV-1 env protein alters the cellular host range and antigenic structure of the virus, J. Virol. ,Vol.68,7689-7696(1994)

[9] M.Ohya; Information theoretical treatment of genes, The Trans. of the IEICE,Vol.E 72,No.5,pp.556-560(1989)

[10] K.Sato,S.Miyazaki and M.Ohya; Analysis of HIV by entropy evolution rate, Amino Acids 14,343-352(1998)

[11] E.C.Holmes,L.Q.Zhang,P.Simmonds,C.A.Ludlam, and A.J.L.Brown; Con-vergent and divergent sequence evolution in the surface envelope glycoprotein of human im

mu-nodeficiency virus type 1 within a single infected patient, Proc.Natl.Acad.Sci.U.S.A.,Vol.89,4835-4839(1992)

[12] T.McNearney,Z.Hornickova,R.Markham,A.birdwell;M.Arens,A.Saah,and L.Ratner; Relationship of human immundeficiency virus type 1 sequence heterogeneity to stage of disease, Proc.Natl.Acad.Sci.U.S.A.,Vol.89,10247-10251(1992)

[13] S.M.Wolinsky,B.T.M.Korber,A.U.Neumann,M.Daniels,K.J.Kunstman,A.J.Whetsell, M.R.Furtado,Y.Cao,D.D.Ho,J.T.Safrit,R.A.Koup; Adaptive Evolution of Human Immnodeficency Virus-Type 1 During the Natural Course of Infection, SCIENCE,Vol.272, pp.537-542(1996)

[14] M.Ohya,S.Miyazaki,Y.Ohsima; A new method of alignment, Viva Origino, Vol. 17, No.3, 139-151(1989)

中温水中におけるＲＮＡポリマーの加水分解反応の速度論：ＲＮＡとリボヌクレアーゼの化学進化に関する考察

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要旨

ＲＮＡのホモおよびコポリマーの加水分解速度定数を60 - 120 ℃，pH 8で$MgCl_2$の非存在下および存在下で決定した．加水分解速度はMg^{2+}イオンによって47 - 290 倍促進され，またＲＮＡの核酸塩基の種類に依存した．ホモポリマー poly(A) と poly(U) は poly(G) および poly(C)，poly(A)・poly(U) 会合体，およびコポリマー poly(A,U) と poly(A,U,C) と比較して不安定であった．poly(A)の加水分解反応速度定数の温度依存性を決定し，リボヌクレアーゼによる加水分解速度定数と比較した．これらの速度論的解析にもとづいて，高温下でのＲＮＡの化学進化および高温下での原始的なリボヌクレアーゼの速度論的な制約について考察した．

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KINETICS OF HYDROLYSIS OF RIBONUCLEOTIDE POLYMERS IN AQUEOUS SOLUTION AT ELEVATED TEMPERATURES : IMPLICATIONS OF CHEMICAL EVOLUTION OF RNA AND PRIMITIVE RIBONUCLEASE

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ABSTRACT

The rate constants of hydrolysis of ribonucleotide homo- and copolymers were determined in the absence and presence of $MgCl_2$ in pH 8 aqueous solution at 60 - 120 °C. The hydrolysis rate was enhanced by Mg^{2+} by 47 - 290 times, which was dependent on the type of nucleotide bases. Homopolymers poly(A) and poly(U) were less stable than poly(G) and poly(C), poly(A)·poly(U) duplex, and copolymers poly(A,U,C) and poly(A,U) at 80 °C. The temperature dependence of the rate constants of poly(A) hydrolysis was determined and compared with the rate constants with ribonucleases. Basing on the kinetic analysis, the possibility of chemical evolution of RNA at high temperatures and a kinetic restriction for the emergence of primitive ribonuclease at high temperatures are discussed.

Key words: RNA hydrolysis - Polynucleotide - Kinetic analysis - RNA world - Origin of life - Hydrothermal reactions - High temperature - Mg^{2+} catalyst - Ribonuclease

INTRODUCTION

The discovery of the catalytic activity of RNA in processing RNA transcripts suggests that RNA-like molecules had a central role in the first life on earth.[1] If this hypothesis is correct then RNA-like molecules accumulated spontaneously under primitive earth conditions. There have been a number of reports of successful studies of the condensation of activated nucleotides to form RNA oligonucleotides,[2-4] while it seems surprising that the decomposition of RNA has little investigated.[5,6] However, both the polymerization and depolymerization rate of RNA are essential to evaluate the accumulation behavior of RNA under the primitive earth conditions.

On the other hand, it has been realized that hydrothermal environments had an important role on chemical evolution of biomonomers and biopolymers.[7-

11) Hence, the investigation of chemical evolution of RNA in hydrothermal conditions is also indispensable. In general, RNA is thought to be labile in aqueous solution at high temperatures. If RNA does not have adequate stability at high temperatures, it should be explained how RNA survived under hydrothermal conditions. Possibly, the RNA world had not been exposed under hydrothermal environments.[12] If these assumptions are not elucidated, otherwise the low stability of RNA becomes a major stumbling block for the RNA world hypothesis. However, little kinetic analysis has been carried out on the hydrolysis of RNA at elevated temperatures.

In this study, we have carried out preliminary study on kinetics of hydrolytic stability of ribonucleotide homo- and copolymers in aqueous solution at elevated temperatures. The rate constants of hydrolysis of the nucleotide polymers were determined in the absence and presence of $MgCl_2$. The activation energy was calculated from the temperature dependence of hydrolysis rate of polyadenylic acid (poly(A)). The hydrolysis rate of polynucleotides at elevated temperatures were compared to that with ribonuclease of modern organisms. The kinetic analysis led to proposals of the possibility of survival of the RNA world at high temperatures.

EXPERIMENTAL

Materials. Poly(A), polyuridylic acid (poly(U)), polyguanylic acid (poly(G)), and polycytidylic acid (poly(C)) were purchased from SIGMA and poly(A)·poly(U) complex were obtained from Seikagaku Corporation (Japan). Copolynucleotides involving adenine, uracil, and cytidine bases were synthesized using polynucleotide phosphorylase (PNPase).[13] PNPase was purchased from SIGMA and Seikagaku corporation. The nucleotide composition of the copolymers was determined by complete hydrolysis with ribonuclease T_2 (RNase T_2). All other reagents used were of analytical grade. Abbreviations for the reagents are as follows; 2'-AMP, adenosine 2'-monophosphate; 3'-AMP, adenosine 3'-monophosphate; 5'-AMP, adenosine 5'-monophosphate; 2',3'-cAMP, adenosine 2',3'-cyclic monophosphate; 3',5'-cAMP, adenosine 3',5'-cyclic monophosphate; HEPES, 2-(4-(2-hydroxyethyle)-1-piperazinyl)ethanesulfonic acid; TBABr, tetrabutylammonium bromide.

Methods. HPLC analyses were performed on a DNA-NPR anion-exchange column (TOSOH Co., Japan) using a gradient of 0.3 - 1.2 M NaCl at pH 9.0 (pH=11.0 for poly(G)) with 0.02 M tris buffer and on a ODS-2 column (GL Science Co., Japan) using a gradient of 0.02 M NaH_2PO_4 with 0.005 M TBABr in aqueous solution at pH 3.5 mixed with 0.02 M NaH_2PO_4 with 0.005 M TBABr in 60 % CH_3OH at pH 3.5. The detection of polynucleotide was carried out at 260 nm for poly(A), poly(A,U), and poly(A)·poly(U), at 271 nm for poly(C), 262 nm for poly(U), 253 nm for poly(G), and 265 nm for poly(A,U,C).

Kinetic analysis of hydrolysis of polynucleotides. Unless noted otherwise, reaction solutions of 0.25 mM polynucleotide containing 0.2 M NaCl, 0.1 M HEPES (pH 8.0) with and without 0.1 M $MgCl_2$ were freshly prepared for each experiment. The temperature of the reaction mixture was controlled by a dry bath

heater. The reaction mixture was allowed to stand at 60 - 120 °C for over 480 h. The sample was quenched immediately after the reaction and analyzed on the anion-exchange HPLC.

The disappearance of phosphodiester bond was monitored to evaluate the hydrolysis rate. The degree of polymerization (length n) of a polynucleotide and its hydrolyzed oligomers were determined basing on the retention time on the anion-exchange HPLC. The HPLC method separates fractions on the basis of the number of negative charges on each oligonucleotide. Thus, it was possible to determine the length n of the nucleotides on the basis of the retention times using authentic oligonucleotides. The normalized value (B_t) of the total number of phosphodiester bond of the products was determined by using equation (1).

$$B_t = \{ \Sigma \text{ (percentage of nucleotide length n) x (n - 1) at t } \} / \{ \Sigma \text{ (percentage of nucleotide length n) x (n - 1) at t=0 } \} \cdots (1)$$

The values of B_t were plotted as a function of time and the rate constant was determined by the computer program SIMFIT.[14] (Terfort and von Kiedrowski 1992). The polynucleotide used in this study involves different length of nucleotide, thus the rate constants indicate approximate values which were the average of different length of polynucleotides. The hyperchromic effect for poly(G) was not corrected since it was analyzed at pH 11 on HPLC[15] and that for other homopolymers were not significant for this kinetic analysis. The compositions and the chain length of the polynucleotides used in the study are summarized in Table 1.

The hydrolysis rate of 2'-AMP, 3'-AMP, 2',3'-cAMP, and 3',5'-cAMP was measured in a buffer solution containing 0.1 M NaCl and 0.05 M HEPES (pH 8.0). The reaction products were analyzed on the reversed-phase HPLC.

Table 1 Chain length and composition of polynucleotides

polynucleotides	mean chain length
poly(A)	330
poly(U)	210
poly(C)	170
oligo(G)[a]	< 15
poly($A_{0.2}U_{0.8}$)[b]	20
poly($A_{0.2}U_{0.4}C_{0.4}$)[b]	40
poly(A)·poly(U)	45

[a] Oligo(G) was used since poly(G) does not dissolve in the buffer solution.

[b] The number shown in parentheses is the ratio of incorporated bases in the polynucleotides.

RESULTS AND DISCUSSION

Kinetics of hydrolysis of polynucleotide

The reactions were carried out at 60 - 120 °C and immediately stopped by freezing, thus the enzymatic contamination of RNase is negligible. The reaction in the presence of $MgCl_2$ was notably faster than that in the absence. Reaction curves and the rate constants were determined for the hydrolysis of polynucleotides (Fig. 1a, 1b, Table 2) in the absence and presence of $MgCl_2$. The hydrolysis rate in the presence of $MgCl_2$ is larger than that in the absence of $MgCl_2$ in which the enhancement of hydrolysis was in the range of 50 - 330 times. The enhancement by Mg^{2+} was not notable in previous studies.[16,17] The reason seems to be due to the fact that previous studies had been carried out at relatively low concentration of Mg^{2+}. Normally, Mg^{2+} ion forms complex with phosphate group of nucleotide rather than nucleotide bases.[18] Thus, the acceleration of hydrolysis in the presence of $MgCl_2$ is presumably due to the complexation of Mg^{2+} with phosphodiester group.

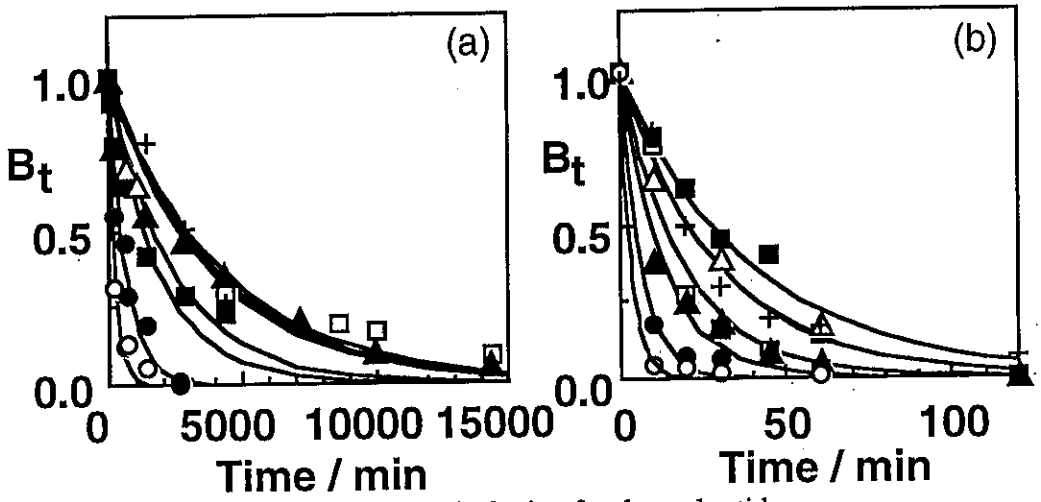

Fig. 1 Reaction curves for the hydrolysis of polynucleotides.
Reaction conditions: [NaCl] = 0.1 M, [HEPES] = 0.05 M, [poly(A)] = 2.5×10^{-4} M, initial pH=7.0, 80 °C. a : $[MgCl_2]$=0 M, b : $[MgCl_2]$=0.1 M.
○ : poly(A), ● : poly(U), □ :poly(C), ■ : poly(G), △ : poly($A_{0.2}U_{0.8}$), ▲ : poly($A_{0.2}U_{0.4}C_{0.4}$), + : poly(A)·poly(U) complex.

Although Mg^{2+} catalyses the hydrolysis of polynucleotides, it does not instantaneously imply that Mg^{2+} caused disadvantage for the survival of RNA under hydrothermal conditions. The accumulation of RNA could be possible in primitive earth conditions if the acceleration of polymerization of RNA by Mg^{2+} was much greater than that of decomposition of RNA. Actually, it has been found that the model reactions of spontaneous formation of RNA are catalyzed by Mg^{2+}.[2,4]

On the other hand, the hydrolysis rate is dependent on the type of nucleotide bases. Moreover, copolymers poly(A,U), poly(A,U,C), and poly(A)·poly(U) complex are more stable than poly(A) and poly(U). This fact may indicate that copolynucleotides are more stable than homopolymers. The rate constants presented in this study involve different length of polynucleotides

so that the difference of the rate constants may reflect the dependence on polynucleotide length. However, it is reasonable that the magnitude of the hydrolysis rate would be approximately independent on length n since their length are long. Since it is thought that both hydrogen bonding and hydrophobic interaction are not effective at elevated temperature,[19] especially for adenine and uracil base pair, the enhancement of stability of copolymers is not understandable by conventional theory basing on hydrogen bonding and stacking interaction. Detail analysis of rate constants for different bases and length of nucleotide will be necessary.

Table 2 Rate constants for the hydrolysis of polynucleotides (s^{-1})

polynucleotides	No Mg^{2+}	$[Mg^{2+}]$ = 0.1 M
poly(A)	$(6.80 \pm 0.46) \times 10^{-5}$	$(5.14 \pm 0.33) \times 10^{-3}$
poly(U)	$(2.60 \pm 0.19) \times 10^{-5}$	$(2.75 \pm 0.14) \times 10^{-3}$
poly(C)	$(3.89 \pm 0.29) \times 10^{-6}$	$(8.67 \pm 0.54) \times 10^{-4}$
poly(G)	$(8.89 \pm 0.82) \times 10^{-6}$	$(4.20 \pm 0.16) \times 10^{-4}$
poly(A,U)	$(6.85 \pm 0.36) \times 10^{-6}$	$(5.58 \pm 0.24) \times 10^{-4}$
poly(A,U,C)	$(4.37 \pm 0.27) \times 10^{-6}$	$(1.27 \pm 0.08) \times 10^{-3}$
poly(A)·poly(U)	$(4.13 \pm 0.24) \times 10^{-6}$	$(5.61 \pm 0.22) \times 10^{-4}$
poly(A), 60 °C	ND	$(1.54 \pm 0.02) \times 10^{-3}$
poly(A), 100 °C	$(1.68 \pm 0.11) \times 10^{-4}$	$(1.08 \pm 0.06) \times 10^{-2}$
poly(A), 120 °C	$(3.83 \pm 0.16) \times 10^{-4}$	ND

[NaCl] = 0.1 M, [HEPES] = 0.05 M, [polynucleotides] = 2.5×10^{-4} M, Temperature : 80 °C, ND : Not determined.

The rate constants of hydrolysis of poly(A) were determined in the range of 60 - 120 °C in the absence and presence of $MgCl_2$ (Table 2). The apparent activation energy (E_{app}) determined from the Arrhenius plot was 50 kJ mol^{-1} in the absence of $MgCl_2$ and 51 kJ mol^{-1} in the presence (Fig. 2). The magnitude of E_{app} is regarded as independent on the Mg^{2+} presence thus the acceleration by Mg^{2+} is considered to be due to the enhancement of the frequency factor by Mg^{2+}. The linearity of the Arrhenius plots supports that the hydrolytic mechanism are not dependent on temperature at 60 - 120 °C.

It was observed that completely hydrolyzed products of polynucleotides contained 3'-AMP, 2'-AMP, 2',3'-cAMP, and adenosine but no 5'-AMP. Further, oligonucleotides having 2'- and 3'-phosphate terminal were detected on the reversed-phase HPLC. These facts indicate that poly(A) was cleaved to form 3'-phosphate terminals through 2',3'-cyclic-AMP as intermediate, which is consistent with previous investigations[20-22] and is similar to enzymatic hydrolysis.[23] The analysis of hydrolysis of 2'-AMP and 3'-AMP shows that the interconversion of 2'-AMP and 3'-AMP occurred through 2',3'-cAMP as intermediate at elevated temperatures. The hydrolysis rate of the monomers was comparable to that of

poly(A) (Table 3). Thus, the rate determining step is considered as cleavage of 3',5'-phosphodiester bond to form 2',3'-phosphodiester terminal, which is also consistent with previous studies.[23]

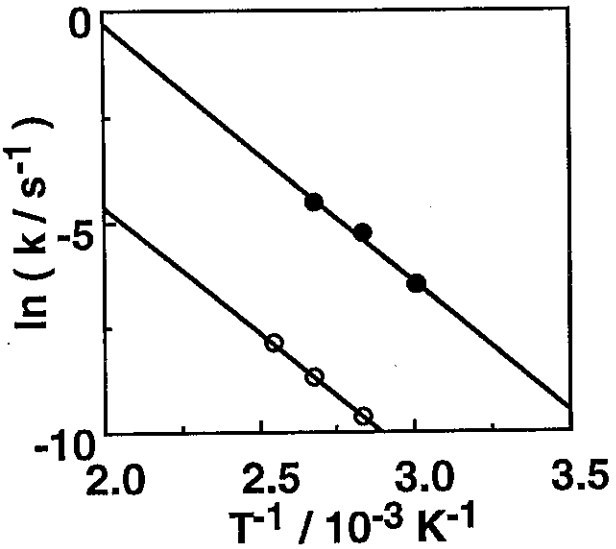

Fig. 2 Arrhenius plots of the rate constants of hydrolysis of poly(A).
● : $[MgCl_2] = 0$ M, ○ : $[MgCl_2] = 0.1$ M. All other conditions are the same as shown in Fig. 1.

Table 3 Rate constants of hydrolysis of cyclic-AMP

cyclic-AMP	Rate constants (s^{-1})
2',3'-cyclic-AMP	4.0×10^{-5}
3',5'-cyclic-AMP	6.0×10^{-6}

Reaction conditions: [NaCl] = 0.1 M, [HEPES] = 0.05 M, [polynucleotides] = 2.5×10^{-4} M, initial pH = 7.0, 120 °C, no $MgCl_2$ added.

Chemical evolution of RNA in hot ocean

Basing on the Arrhenius plots, the rate constant of hydrolysis of poly(A) is estimated to be 4.2×10^{-4} s^{-1} at 37 °C (half-life ($t_{1/2}$), 28 min), 1.3 s^{-1} at 250 °C ($t_{1/2}$, 0.5 s), and 12 s^{-1} at 374 °C ($t_{1/2}$, 0.06 s) in the presence of 0.1 M $MgCl_2$. This fact may indicates that rapid decomposition of phosphodiester bond possibly interfered the accumulation of RNA under hydrothermal conditions on the primitive earth. Actually, basing on the study of the stability of ribose in aqueous solution at elevated temperatures,[6] in which the decomposition rate is much slower than that of phosphodiester bond, it was postulated that the RNA world emerged in cool ocean rather than hot.[12] Although this speculation may be correct, the accumulation of RNA is determined by both the rate of polymerization and depolymerization. Thus, it is unreasonable to speculate straightforwardly that the RNA world could not survive under hydrothermal environments. If the formation rate of RNA under the primitive earth conditions

was so rapid compared to the deformation rate, the RNA world could survived. In order to evaluate this assumption, abiotic formation of RNA at elevated temperatures is being investigated at present.

On the other hand, the comparison of the hydrolysis rate without enzymes and that with primitive enzyme is valuable to consider the emergence of the RNA world. The reason is that the biological reactions in modern organisms are universally controlled by enzymes so that it is reasonable to assume that the prebiotic reactions were being controlled by primitive enzymes.

One of the essential factor which sets up enzymatic activity is that the rate with enzyme must be faster than the rate without enzyme. Actually, the catalytic enhancement (k_{cat} / k_{non}) by modern enzymes[24] is generally in the range of 10^5 - 10^{17}. In other words, the large enhancement ability is required for the modern enzymes to control biological metabolism with adequate accuracy. Thus, ribonuclease-like activity could not appear at least unless the catalytic rate with primitive RNase became larger than that without RNase. That is to say, the hydrolysis rate with primitive RNase should be faster than that without primitive RNase at time of the origin of life. Recent analyses of archaeal and bacterial phylogenetic trees suggest that the last common ancestor of all present-day organisms had nature of hyperthermophiles.[11,25,26] Actually, there are hyperthermophilic bacteria and archaea which are cultured over 100 °C and even which could survive at 250 °C,[27] the rate with RNases should be much faster than that without RNase.

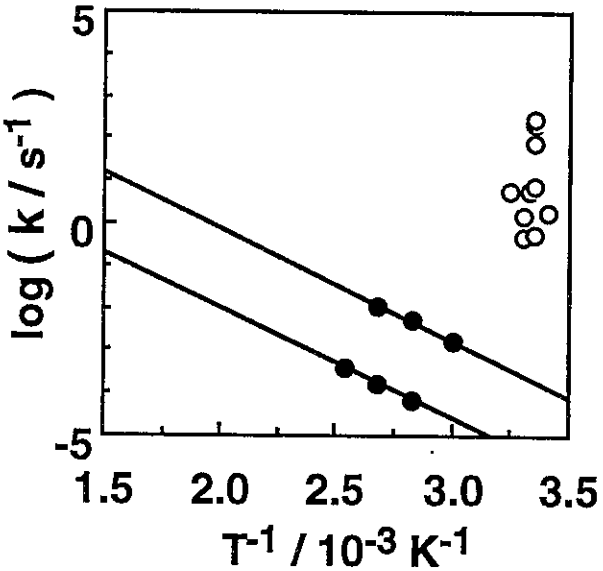

Fig. 3 Comparison of hydrolysis rate with and without RNases and extrapolation of RNase activity to time at the origin of life.
Open circles : V_{max} with RNases[28-37]. Closed circle : Rate constants determined in this study.

The comparison of the rate constants of hydrolysis of polynucleotide with and without RNase are demonstrated in Fig. 3.[28-37] The hydrolysis rate constants with RNases, which are the values of V_{max}, are much faster than those without RNases at low temperatures. There is no geological evidence on the

temperature of the primitive earth,[38] however, the nature of primitive enzymes on the last common ancestor is possibly estimated from a simple extrapolation of the enzymatic activity of modern hyperthermophiles to time of the origin of life. Since the rate with RNase at elevated temperatures is less known,[37] it is not possible to extrapolate the rate of RNases. However, kinetics on other kind of enzymes indicate that the catalytic enhancement and Michaelis-Menten values are not very sensitive with temperature.[39-43] Thus, the rate with primitive RNases could be as fast as that with modern enzymes. The large gap between the rate with and without RNases supports possibility that RNase activity could emerge even at high temperatures.

Consequently, the speculation in this study emphasizes that the hydrolysis without RNase at elevated temperatures is not straightforwardly regarded as too fast as to destroy the RNA world. In order to evaluate this hypothesis, kinetics on the formation of RNA and the activity of primitive enzymes at elevated temperatures are being investigated to draw the evolutionary history of the RNA world and primitive RNase.

CONCLUSIONS

The rate constants of hydrolysis of polynucleotides were determined at elevated temperatures. It was found that Mg^{2+} enhances the hydrolysis and the rate constants seem to be dependent on the type of nucleotide base. Basing on the comparison of the hydrolysis rate with and without enzyme, it is regarded that the hydrolysis rate without enzyme at elevated temperatures are much slower than that with RNases. These evaluations imply possibility that RNA world could survive at higher temperatures.

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REFERENCES

1) Cech, T. R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4360-4363.
2) Lohrmann, R. and Orgel, L. E. (1980) *J. Mol. Biol.* **142**, 555-567, **123**, 127-149.
3) Sawai, H., Kuroda, K. and Hojo, H. (1989) *Bull. Chem. Soc. Jpn.* **62**, 2018-2023.
4) Ferris, J. P. and Ertem, G. (1992) *Origins Life Evol Biosphere* **22**, 369-381.
5) White, R. H. (1984) *Nature* **310**, 430-432.
6) Larralde, R., Robertson, M. P. and Miller, S. L. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 8158-8160.
7) Campbell, L. L. and Pace, B. (1968) *J. Appl. Bact.* **31**, 24-35.

8) Yanagawa, H. and Egami, F. (1978) *Proc. Jpn. Acad. Ser. B* **54**, 331-336.
9) Miller, S. L. and Bada, J. L. (1988) *Nature* **334**, 609-611.
10) Holm, N. G. (ed) (1992) *Origins Life Evol Biosphere* **22**, 5-242.
11) Oshima, T. (1994) *Trans. Mat. Res. Soc. Jpn.* **19B**, 1069-1077.
12) Miller, S. L. and Lazcano, A. (1995) *J. Mol. Evol.* **41**, 689-692.
13) Steiner, R. F. and Beers, Jr. R. F. (1958) *J. Polymer Sci.* **30**, 17-28.
14) Terfort, A. and von Kiedrowski, G. (1992) *Angew. Chem. Int. Ed.* **31**, 654-656.
15) Kanavarioti, A., Bernasconi, C. F., Alberas, D. J. and Baird, E. E. (1993) *J. Am. Chem. Soc.* **115**, 8537-8546.
16) Butzow, J. J. and Eichhorn, G. L. (1965) *Biopolymer* **3**, 95-107.
17) Wintermeyer, W. and Zachau, H. G. (1973) *Biochim. Biophys. Acta* **299**, 82-90.
18) Taqui Khan, M. M. and Martell, A. E. (1962) *J. Am. Chem. Soc.* **84**, 3037-3041.
19) Turner, D. H. and Bevilacqua, P. C. (1993) in Gesteland, R. F. and Atkins, J. F. (ed) The RNA World. Cold Spring Harbor Press, New York, p 447-464.
20) Bacher, J. E. and Kauzmann, W. (1952) *J. Am. Chem. Soc.* **74**, 3779-3786.
21) Eigner, J., Boedtker, H. and Michaels, G. (1961) *Biochim. Biophys. Acta* **51**, 165-168.
22) Eichhorn, G. L. and Butzow, J. J. (1965) *Biopolymers* **3**, 79-94.
23) Koike, T. and Inoue, Y. (1972) *Chem. Lett.* 569-572.
24) Radzicka, A. and Wolfenden, R. (1995) *Science* **267**, 90-93.
25) Pace, N. R. (1991) *Cell* **65**, 531-533.
26) Forterre, P. (1995) *Cell* **85**, 789-792.
27) Baross, J. A. and Dening, J. W. (1983) *Nature* **303**, 423-426.
28) Witzel, H. and Barnard, E. A. (1962) *Biochem. Biophys. Res. Comm.* **7**, 296-299.
29) Batt, C. W. and Houck, J. C. (1964) *Biochem* . **89**, 90-94.
30) Sato, S., Uchida, T. and Egami, F. (1966) *Arch. Biochem. Biophys.* **115**, 48-52.
31) Irie, M. and Hosokawa, S. (1971) *J. Biochem.*. **70**, 301-309.
32) Williams, R. K. and Shen, C. (1972) *Arch. Biochem. Biophys.* **152**, 606-612.
33) Li, J. R. T. and Walz, Jr. F. G. (1974) *Arch. Biochem. Biophys.* **161**, 227-233.
34) Walz, Jr. F. G., Osterman, H. L. and Libertin, C. (1979) *Arch. Biochem. Biophys.* **195**, 95-102.
35) Osterman, H. L. and Walz, Jr. F. H. (1979) *Biochem.* **18**, 1984-1988.
36) Irie, M., Mikami, F., Monma, K., Ohgi, K., Watanabe, H., Yamaguci, R. and
37) Kanaya, S. and Itaya, M. (1992) *J. Biol. Chem.* **267**, 10184-10192.
38) Kasting, J. F. (1993) *Science* **259**, 920-926.
39) Yamanaka, K. (1968) *Biochim. Biophys. Acta* **151**, 670-680.
40) Danno, G. (1970) *Agr. Biol. Chem.* **34**, 1805-1814.
41) Van Bastelaere, P., Vangrysperre, W., Kersters-Hilderson, H. (1991) *Biochem. J.* **278**, 285-292.
42) Adams, M. W. W. (1993) *Ann. Rev. Microbiol.* **47**, 627-658.
43) Adams, M. W. W. and Kelly, R. M. (1994) *Bioorg. Med. Chem.* **2**, 659-667.

THE EFFECT OF X-RAY IRRADIATION ON αA-CRYSTALLIN

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Abstract

We have studied the aspartyl (Asp)-151 residue of αA-crystallin was inverted to biologically uncommon D-isomer in human, bovine and mouse eye lenses in spite of the difference of species with age. Our previous study demonstrated that UV-B irradiation induced cataract, aggregation of lens protein, and the specific racemization at Asp-151 residue in αA-crystallin of rat lens [1]. We report here that X-ray irradiation (10 Gy) induced increase of the hydrophobicity of αA-crystallin at 8 weeks after X-ray irradiation and the mature cataract and high molecular weight protein (HMW) at 12 weeks after X-ray irradiation, however, the racemization of Asp-151 residue was rather suppressed by X-ray irradiation.

1. Introduction

The mammalian lens soluble proteins consist mainly of α-,β- and γ-crystallin. α-Crystallin is the most abundant protein and comprised of two highly homologous 20k Da subunit, αA and αB. The two subunits associate non-covalently to form aggregate with average molecular weight 800 kDa. Since the lens protein is metabolically inert, numerous posttranslational modifications such as racemization[1-6], isomerization [1,2,4,6], deamidation [7-10],

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truncation [3,11-15], phosphorylation [16], oxidation [2,6,17], crosslinking in intramolecular disulfide bonding [18,19], glycation [20], acetylation [21], and crosslinking [22] have been reported with aging or cataract formation.

Swamy et.al. reported that the amount of high molecular weight protein (HMW) increased and hydophobicity of αA-crystallin increased (formation of αAm-crystallin) in soluble fraction in aged human lens [23]. Recently, we also observed that UV-B irradiation induced the increase of HMW and the opacification of rat lenses [1]. These results suggested a correlation between opacification and the increase of HMW.

In previous studies, we have reported that two aspartyl residues (Asp-58 and Asp-151) of αA-crystallin in human [2,3] and Asp-151 residue of αA-crystallin in mouse [4] were inverted to D-Asp and the relative amount of D-Asp increased with age. Recently we found that UV-B irradiation induced the racemization of the Asp-151 residue in αA-crystallin obtained from the lens of young rats [1]. In addition, many studies of effects of X-ray irradiation on lens proteins have indicated that irradiation seems to promote the changes similar to those seen in the aging process. For instance, X-ray irradiation induced HMW formation and irregularly shaped giant aggregates in lens fibers, which were an accelerated aging process [24].

The present study was focused on a possible correlation between lens opacity, HMW formation and the racemization of the Asp-151 residue in αA-crystallin.

2. MATERIALS AND METHODS

2.1 X-ray irradiation

Female ICR mice, 4 weeks of age were use. They were divided into two groups, normal control and X-ray irradiation group. For irradiation the head was exposed to 1000 rad (10 Gy) of X-rays with the following exposure factors: 130 kV, 8 mA, 0.5 mm aluminum filter (built in), target distant 30.0 cm (skin-focus) and rate of 188.6 rad/min. Each mouse was immobilized in a holder consisting of a 50 ml plastic centrifuge tube which had been cut in half length wise with the cut surface glued to a flat Lucite plate. The open end was closed with a plug. The head was rested in the conical end which was perforated. The trunk of the animal from the cranium was shielded

with 1-mm- thick lead.

2.2 Purification of αA-Crystallin from Mouse Lens

X-ray irradiated and control lenses (40 in each sample) were homogenized in 50 mM NaCl and 1 mM EDTA/50 mM Tris-HCl buffer (pH 7.4). The proteins were fractionated into water-soluble and water-insoluble fractions by centrifugation at 15000 g for 20 min at 4°C. The water-soluble fraction was applied to a TSK G4000 SW XL (7.8 X 300 mm, Tosoh, Tokyo, Japan) column which were equilibrated with 0.1M Na_2SO_4 /50mM phosphate buffer (pH7.5) and the α-crystallin fraction and HMW fraction were collected. αA-Crystallin was purified by reverse-phase (RP)- HPLC using a C4 column in accordance with the procedure of our previous study [4].

2.3 Enzymatic Digestion and Isolation of Peptides

αA-Crystallin was digested with trypsin for 20 h at 37°C in 0.1 M Tris-HCl buffer, pH 7.6, at an enzyme-to-substrate ratio of 1:50 (mol/mol). The resulting tryptic (T) peptides were separated by RP-HPLC as previously described [4].

2.4 Determination of D/L ratio of Amino Acids

All glassware was baked at 500°C for 3 h. Peptide samples were lyophilized in tubes and were hydrolyzed with gas-phase 6N HCl for 7 h at 108°C(PicoTag Work Stations, Waters, Tokyo). The hydrolysates were evaporated under reduced pressure. After hydrolysis, the samples were derivatized with o-phtalaldehyde (OPA) and *n-tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys) to form diastereoisomers. The determination of the D/L ratio of amino acids was performed by RP-HPLC with a C18 column (Nova-Pak ODS, 3.9 x 300 mm, Waters Japan) using fluorescence detection (344 nm excitation wavelength and 433 nm emission wavelength) according to Hashimoto et al. [25]. Elution was carried out with a linear gradient of 5-47% acetonitrile plus 3% tetrahydrofuran in 0.1 M acetate buffer (pH 6.0) for 120 min at a flow rate of 0.8 ml/min, at 30°C.

2.5 Amino Acid Sequence Analysis

Amino acid sequences were determined using Edman degradation on a pulsed-liquid protein sequencer, equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer (Applied Biosystems 476A/120A, Foster City, CA, U.S.A).

2.6 Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry

All spectra were obtained using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer operated with nitrogen laser (Kompact MALDI IV, Shimadzu Corporation, Japan) as previously described (3). The data were collected in reflection mode as signals of positive ions. The sample peptide (0.5 μl) was added to an equal volume (0.5 μl) of the matrix solution on the plate, and then dried. Each sample was present at a level of a few pmol level per spot.

3. RESULTS

3.1 Cataract formation by X-ray Irradiation

Lenses of the control group of mice, ages one day to 18 months were transparent as determined by slit lamp examination. As shown in Fig.1, X- ray irradiation induced lens opacities were first detected in the posterior subcapular region by slit lamp 4 weeks after exposure to X-ray (b). Plaque-like opacities were detected in the posterior subcapular region 8 weeks after irradiation (c). Mature cataract appeared more than 12 weeks after irradiation (d).

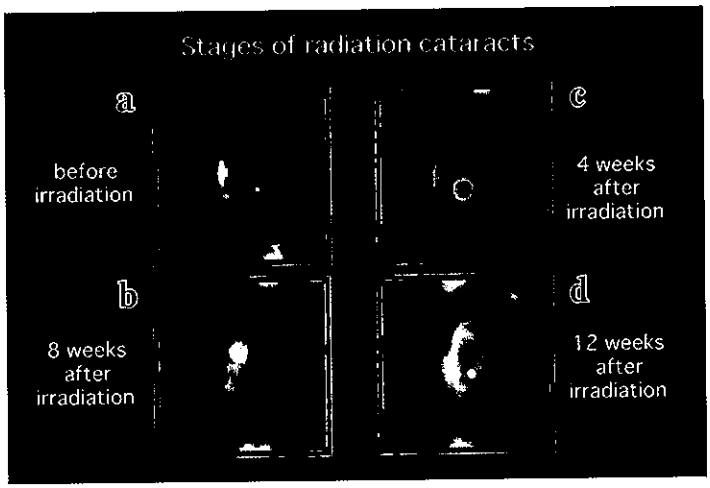

Figure 1
Photograpy of different stage of lenses. (a) before irradiation, (b)4 weeks after X-ray irradiation, (c) 8 weeks after X-ray irradiation, (d) 12 weeks after X-ray irradiation. Mature cataract appeared 12 weeks after X-ray irradiation.

3.2 Induction of HMW Formation by X-ray irradiation

Figure 2a shows a typical gel permeation chromatography (GPC) profile of water-soluble(WS) fraction obtained from 16-week-old mice (non irradiated mice) lens as control. The WS protein composed of α-, β-, γ-crystallin and HMW was not observed. As shown in Fig. 3a, the GPC profile of the lenses 8 weeks after X-ray irradiation (12-week-old) was the same as that of control. However, in the lenses of 12 weeks after irradiation (16-week-old), the amounts of HMW and α-crystallin greatly increased and γ-crystallin decreased as shown in Figure 4a.

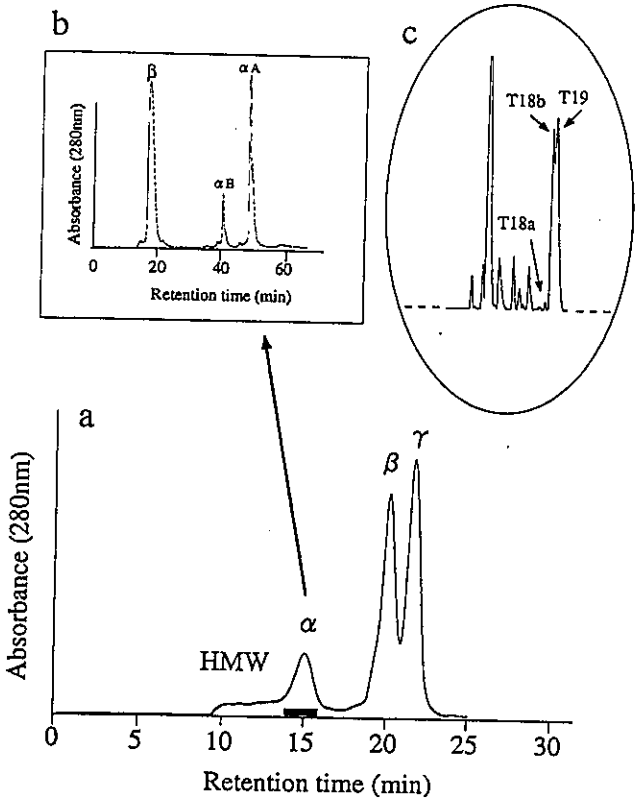

Figure 2
a) Gel permeation chromatography (GPC) profile of water soluble (WS) lens proteins, obtained from 16-week-old mice, with a TSK G4000 SW XL column. 7.8 x 300 mm, (Tosoh, Tokyo, Japan). The column was equilibrated and sample eluted with a buffer consisting of 0.1M potassium phosphate, 0.1M Na_2SO_4, pH7.4) Flow rate : 0.8 ml/min. Detection : 280 nm. b) Reverse phase(RP)-HPLC of α-Crystallin obtained from the fractionation shown in the lower Figure. Column : C4 column (Vydac protein C4, 250 x 4.6 mm) Eluent A: 1% trifluoroacetic acid in water; Eluent B: 1% trifluoroacetic acid in acetonitrile. Gradient : 32% B to 45% B in 30 min, 45% B to 55% B in 8 min, isocratic at 55% B for 7 min, a steep increase from 55% B to 75% B in 4min. isocratic at 75% B for 15min. Flow rate : 1 ml/min. Detection : 280 nm c) Partial RP-HPLC elution profiles of tryptic peptides of αA-crystallin obtained from 16-week-old lenses. Column : C18 column (TSK gel ODS-80TM: 250 x 4.6 mm) Eluent A: 0.1% trifluoroacetic acid in water; Eluent B: 0.1% trifluoroacetic acid in acetonitrile. Gradient : 0-40% B in 150 min. Flow rate : 0.8 ml/min. Detection : 215 nm.

Fig. 2b and Fig. 3b shows RP-HPLC chromatograms of α-crystallins of non-irradiated and 8 weeks after X-ray-irradiated mouse lenses, using a C4 column, respectively. Fig 4b and 4c shows RP-HPLC chromatograms of the HMW protein and α-crystallin obtained from lenses at 12 weeks after X-ray irradiation. As described in the previous study [4], we have identified, β-crystallin(the first peak), αB-crystallin (the second one), αA-crystallin (αA and αAm, the third and forth) by SDS-PAGE (data not shown). We designated αA and αAm (Fig.3b and Fig.4b and Fig.4c) according to Swamy et al. [23] because these profiles were similar with that of α-crystallin obtained from aged human lens that they had reported. While the RP-HPLC chromatograms of α-crystallin obtained from 8 weeks and 12 weeks after irradiation were very similar (Fig 3b and Fig 4c), the chromatogram of HMW fraction of 12 weeks after irradiation (Fig. 4b) was quite different, that is αAm was dominant, αA were not observed, and αB-crystallin decreased. αAm was eluted at high concentration (almost 70%) of acetonitrile on the RP-HPLC, therefore, the hydrophobicity of αAm-crystallin must be higher than that of αA-crystallin.

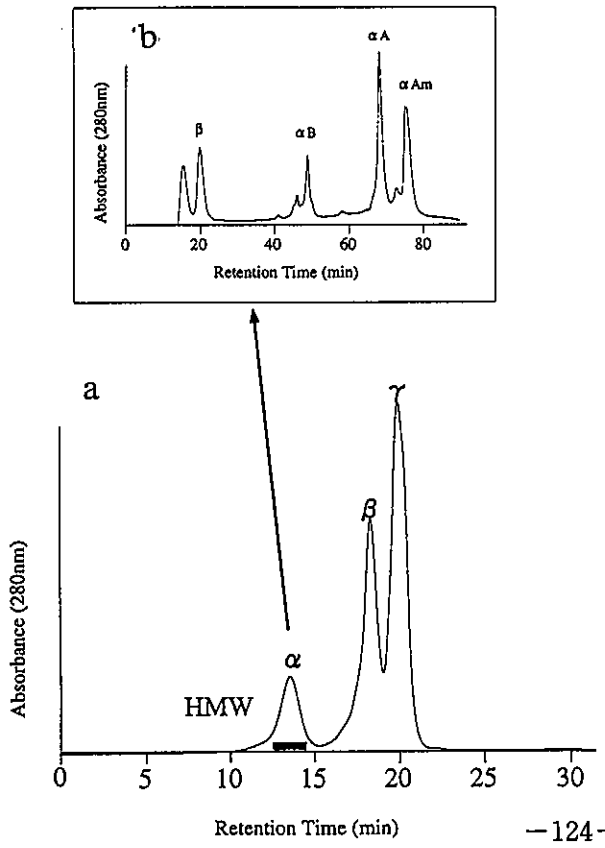

Figure 3
a): Gel permeation chromatography (GPC) profile of WS lens proteins, obtained from 8 weeks after X-ray irradiation(12-week-old mice). The sample elution was performed as described in Figure 2 a.
b): RP-HPLC of α-Crystallin fraction shown in Figure 3a. The sample elution was performed as described in Figure 2 c.

The effect of X-ray on the racemization of Asp-151 residue of αA-crystallin

The following samples were subjected to tryptic digestion and analyzed by RP-HPLC using a C18 column; i) non irradiated αA-crystallin of 16-week-old mouse (αA in Fig.2b), ii) αA and αAm of 8 weeks after X-ray irradiation (αA and αAm in Fig 3b), iii) αA of 12 weeks after irradiation (Fig.4c), iv) αAm obtained from HMW of 12 weeks after irradiation. (Fig.4b). All T18 peptides obtained from irradiated lenses were separated into two peaks (T18a and T18b) as well as control (data not shown). The sequence analysis of T18 peptides obtained from all samples gave the same expected sequence, VQSGLD[151]AGHSER, which indicate that the linkage of the Asp-151 residues in both T18a and T18b are the same α-type (Table I). The results of MS analysis of all T18a and T18b peptides were consistent with the theoretical mass as control (Table I). These results were coincide with our recent results of aged mouse lens [4].

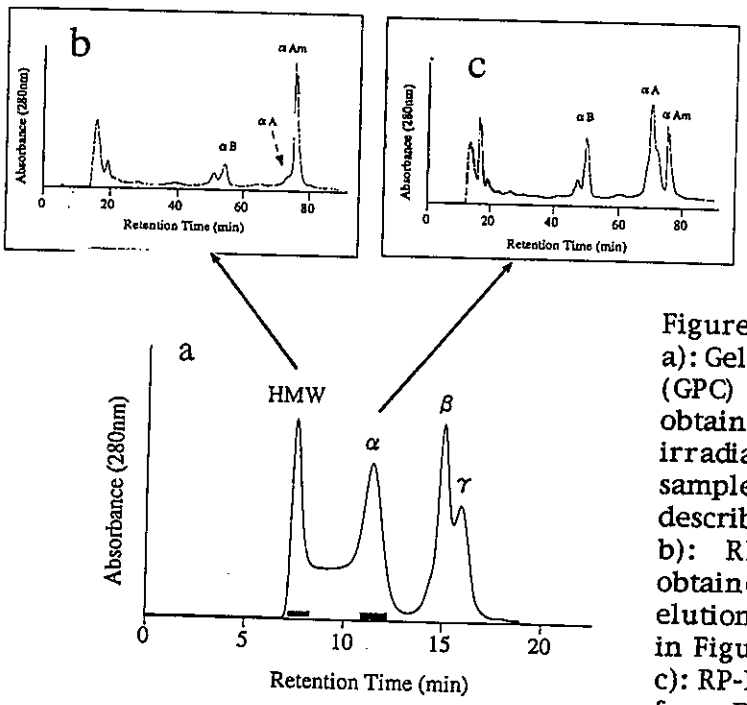

Figure 4
a): Gel permeation chromatography (GPC) profile of WS lens proteins, obtained from 12 weeks after X-ray irradiation (16-week-old mice). The sample elution was performed as described in Figure 2 a.
b): RP-HPLC of HMW fraction obtained from Figure 4a. The sample elution was performed as described in Figure 2 b.
c): RP-HPLC of α-Crystallin obtained from Figure 4a. The sample elution was performed as described in Figure 2 c.

Among all the Asp residues, only the Asp-151 residues in T18a peptides were racemized. The D/L ratio of Asp-151 in T18a were all higher (0.1<D/L) than that in T18b which were 0.02 to 0.06 (Table I). At 8 weeks after irradiation, the D/L ratio of the Asp-151 residues of T18a in αA (0.61) was almost the same as control (0.57), whereas that in αAm decreased to 0.11. At 12 weeks after irradiation, the D/L ratio of Asp-151 in αA decreased dramatically (0.18) for 4 weeks. The D/L ratio of Asp-151 in αAm of both α fraction and HMW were lower (around 0.1) than that in αA.

Table I Characterization of T18 peptide (VQSGLD[151]AGHSER, M+H+=1255.6) of αA-crystallin obtained from lenses 16-week-old (control), 8 weeks and 12 weeks after X-ray irradiation.

Sample	Peptide	Asp151 D/L ratio	$M+H^+$ (Observed)	Sequence (Observed)	Asp151 Linkage	Relative Amount (T18a/T18b)
Control 16-week-old	T18a	0.57	1256.4	VQSGLDAGHSER	α	0.030
	T18b	0.02	1256.9	VQSGLDAGHSER	α	
8W αA (12-week-old)	T18a	0.61	1255.9	VQSGLDAGHSER	α	0.011
	T18b	0.03	1256.2	VQSGLDAGHSER	α	
8W αAm (12-week-old)	T18a	0.11	1255.8	VQSGLDAGHSER	α	0.210
	T18b	0.03	1256.7	VQSGLDAGHSER	α	
12W αA (16-week-old)	T18a	0.18	1255.5	VQSGLDAGHSER	α	0.115
	T18b	0.02	1256.3	VQSGLDAGHSER	α	
12W-HMW αAm (16-week-old)	T18a	0.11	1255.7	VQSGLDAGHSER	α	0.055
	T18b	0.06	1256.8	VQSGLDAGHSER	α	

As shown in Table II, the relative amount of T18a against T18b much higher in αAm(0.21) than αA(0.011) at 8 weeks after irradiation. At 12 weeks after irradiation, the relative amount of T18a in αA increased to 0.115. The yield of αA-crystallin greatly decreased at 12 weeks after irradiation and the relative amount of αAm against αA in α-fraction decreased almost half during 4 weeks

(from 8 weeks after irradiation (αAm:αA=0.77:1) to 12 weeks after irradiation (αAm:αA=0.35:1). Therefore, we could not obtain enough amount of αAm crystallin and could not compare the data of αAm in αA fraction at 12 weeks after irradiation with other data.

Table II Summary of the results from lenses obtained from X-ray irradiated and control and mice lenses.

Sample	Cataract	HMW	αAm	D/L of Asp-151 in T18a in αA	in αAm	Relative Amount (T18a/T18b) αA	αAm
Control							
16-week-old	-	-	-	0.57	-	0.030	-
After X-ray irradiation							
8 weeks	-	-	+	0.61	0.11	0.011	0.210
12 weeks	+++	++	++	0.18	0.11(HMW)	0.115	0.055(HMW)

- : not detected
Each point represents the average of 20 lenses.
The mean variation for duplicate determinnation for each point was ±3%.

4. DISCUSSION

The effect of X-ray on HMW and αAm formation

In this study, X-ray irradiation induced cataract formation with promoting the increase of αAm-crystallin and HMW, which are seen in the extreme aged samples [2,3]. These results are also consistent with UVB-irradiated rat lens[1]. In aging, there is also a marked shift in the distribution of the crystallins to high molecular weight proteins, which were mainly consist of αAm in human lenses[23]. In those previous reports, both αAm appearance and HMW formation were observed at the same time. To the contrary, as summarized in Table II and Fig. 5, αAm-crystallin, more hydrophobically modified αA, appeared prior to HMW formation at 8 weeks after X-ray irradiation, only when plaque-like cataract was detected. Later on, when mature cataract was observed at 12 weeks after irradiation, HMW protein remarkably increased, while such modification of αA-crystallin were not found at least until 20-week-old non irradiated lens[4]. This fact indicates that the increase of hydrophobicity in αA-crystallin is the trigger of HMW formation and lens mature opacificaton. In the lenses obtained from 12 weeks after irradiation, the yield of α-crystallin were greatly decreased to almost half of that of control and 8 weeks after irradiation(data not shown). This observation was coincide with the report that in the most

advanced cases of nuclear cataract, the insoluble fraction can represent more than 30% of total lens protein[26] and indicated that some population of α-crystallin might precipitate as insoluble protein. The increase of insoluble protein is closely related to the cataract formation and α-crystallin had been reported to exist in the insoluble protein by analyzing tryptic peptides [27,28]. The insolubilization of α-crystallin is considered to be mainly caused by crosslinking of proteins. αA-crystallin might occur the conversion from the native soluble protein structure to more hydrophobic by surface conformational change, therefore aggregate with those hydrophobic interaction and become very large aggregate which is not able to be soluble.

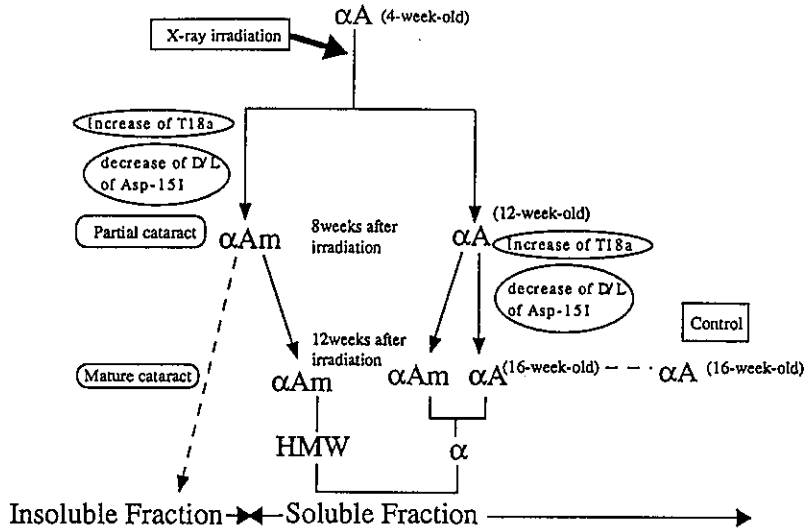

Figure 5
The schematic representation of this experiment of X-ray irradiation on mice lenses.

The Effect of X-ray on the racemization of Asp-151

In our previous studies, both of mouse lenses and UVB irradiated rat lenses, we observed 2 peaks of T18 peptide of αA-crystallin and the D/L ratio of T18a (the former peak) were always higher than that of T18b. Concerning about the D/L ratio of two peaks of T18, we had the same results that the D/L ratio of T18a were always higher than that of T18b in X-ray-irradiated lenses. However, the D/L ratio of T18a in αAm at 8 weeks after irradiation and even in αA at 12 weeks after irradiation rather decreased, whereas the D/L ratio of T18a kept high level in non-irradiated

lenses during aging[4]. This results were opposite to UV B irradiated rat even though rat and mouse has the same αA-crystallin sequence. The difference might be due to the material condition, that is, the difference between the lens culture (UVB irradiated rat) and intact animal eyes (X-ray irradiated mouse), or high order structure related to the interaction among other crystallins such as αB-crystallin and β-crystallin in which there are 3 and 15 amino acid substitutions respectively, or difference mechanism of opacification induced by UVB and X-ray such as energy level.

Furthermore, X-ray irradiation promoted the increase of amount of T18a in αAm over 20 times of that in αA at 8 weeks after irradiation. Since the surface hydrophobicity of αAm seemed higher than that of αA, the increase of T18a might cause the microenvironmental configurational change which expose the hydrophobic region to surface of αA-crystallin.

The fact that X-ray irradiation induced αAm which has low D/L ratio in early stage of cataract and also induced the decreased of the D/L ratio of αA may be due to partial unfolding of the native higher order structure around T18 which need to keep high D-Asp-151 level and this may be crucial for transparency.

REFERENCES

1. Fujii, N. , Momose, Y. Ishibashi, Y., Uemura, T., Takita, M. and Takehana, M. (1997) Specific racemization and isomerization of the aspartyl residue of αA-crystallin due to UV-B irradiation. Exp. Eye Res. 65, 99-104
2. Fujii, N., Satoh, K., Harada, K., and Ishibashi, Y. (1994). Simultaneous stereoinversion and isomerization at specific aspartic acid residues in αA-crystallin from aged human lens. J. Biochem. 116, 663-669.
3. Fujii N., Momose Y., Yamasaki M., Yamagaki T., Nakanishi H., Uemura T., Takita M., Ishii N. (1997) The conformation formed by the domain after alanine-155 induces inversion of aspartic acid-151 in αA-crystallin from aged human lenses. Biochem. Biophys. Res. Comm. 239:918-923
4. Momose, Y., Fujii, N., Kodama, T., Yamagaki, T., Nakanishi H., and Kodama, M. (1998) The Specific racemization of aspartyl residue of αA-crystallin obtained from aged mouse lenses. Viva Orig. 26:329-340

5. Groenen, P.J.T.A., van den IJssel, P.R.L.A., Voorter, C.E.M., Bloemendal, H., and de Jong, W.W. (1990). Site-specific racemization in aging αA-crystallin. FEBS Lett. 269, 109-112.
6. Fujii, N., Ishibashi, Y., Satoh, K., Fujino, M., and Harada, K. (1994). Simultaneous racemization and isomerization at specific aspartic acid residues in αB-crystallin from aged human lens. Biochim. Biophys. Acta. 1204, 157-63.
7. Voorter, C.E.M., de Haard-Hoekman, W.A., van den Otelaar, P.J.M., Bloemendal, H., and de Jong, W.W. (1988) Spontaneous peptide bond cleavage in aging α-crystallin through a succinimide intermediate. J. Biol. Chem, 263, 19020-23.
8. Takemoto L.J.(1998) Quantitation of asparagine-101 deamidation from alpha-A crystallin during aging of the human lens. Curr. Eye. Res.17:247-250.
9. Lund A.L., Smith J.B., and Smith D.L. (1996) Modification of the water-insoluble human α-crystallins. Exp. Eye Res. 63:661-672
10. Lampi K.J., Ma Z., Hanson S.R.A., Azuma M., Shih M., Shearer T.R., Smith D.L., Smith J.B., and David L.L. (1998) Age-related changes in human lens crystallins identified by two-dimensional electrophoresis and mass spectrometry. Exp.Eye Res. 67: 31-43
11. Takemoto L. and Emmons T.(1991) Truncation of αA-crystallin from the human lens. Exp.Eye.Res.53:811-813
12. Emmons T. and Takemoto L.(1992) Age-dependent loss of the C-terminal amino acid from alpha crystallin. Exp.Eye Res. 55:551-554
13. Takemoto L.J.(1998) Quantitation of specific cleavage sites at the C-terminal region of alpha-A crystallin from human lenses of different age. Exp.Eye Res.66:263-266
14. Kamei, A., Iwase, H. and Masuda, K. (1997) Cleavage of amino acid residues from the αA- and αB-crystallin in human crystallin lens during aging. *Biochem. Biophys. Res. Comm* 231, 373-378
15. Inomata, M., Nomura, K., Takehana, M., Saido. T.C., Kawashima, S., Shumiya, S. (1997) Evidence fro the involvement of calpain in cataractogenesis in Shumiya cataract rat (SCR). *Biochim. Biophys. Acta* 1362, 11-23
16. Takemoto, L. (1996) Differential phosphorylation of αA-crystallin in human lens of different age. Exp. Eye Res. 62, 499-504.
17. Takemoto, L., Horwitz and Emmons, T. (1992) Oxidation of N-terminal methionine of lens αA-crystallin. Curr. Eye Res. 11, 651-655
18. Miesbauer, L. R., Xuanjing, Zhou, Yang, Zhucheng, Yang, Zhiying,

Sun, Yiping, Smith, D.L., and Smith, J.B. (1994) Posttranslational modifications of water-soluble human lens crystallins from young adults. J. Biol. Chem. 269, 12494-12502.

19. Takemoto, L. (1996) Increase in the intramolecular disulfide bonding of alphaA-crystallin during aging of the human lens. Exp. Eye Res. 63, 585-590
20. Swamy, M.S., Abraham, A., and Abraham, E.C. (1992) Glycation of human lens proteins: preferential glycation of αA subunits. Exp. Eye Res. 54, 337-45
21. Lin, P.P., Barry, R.C., Smith, D.L., Smith, J.B. (1998) In vivo acetylation identified at lysine 70 of human lens αA-crystallin. Protein Sci. 7, 1451-1457.
22. Chen, Y.C., Reid, G.E., Simpson, R.J., and Truscott, J.W. (1997) Molecular evidence for the involvement of alpha crystallin in the colouration/crosslinking of crystallins in age-related nuclear cataract. Exp. Eye Res. 65, 835-840
23. Swamy, M.S. and Abraham, E.C. (1991). Reverse-phase HPLC analysis of human α-crystallin. Curr. Eye Res. 10, 213-220
24. Liem-The, K., Stols, A.L.H., Jap, P.H.K., and Hoenders, H.J. (1975) X-ray induced cataract in rabbit lens. Exp. Eye Res. 20,317-328.
25. Hashimoto, A., Nishikawa, T., Takahashi, K., and Hayashi, T. (1992). Determination of free amino acid enantiomers in rat brain and serum by high-performance liquid chromatography after derivatization with N-*tert.*-butyl-oxycarbonyl-L-cysteine and *o*-phthaldialdehyde. J. Chromoatogr. 582, 41-48
26. Truscott, R.J.W. and Augusteyn, R.C. (1977) Changes in human lens proteins during nuclear cataract formation. Exp.Eye Res. 24, 159-170
27. Lund, A.L., Smith, J.B. and Smith, D.L. (1996) Modification of the water-insoluble human lens α-crystallin. Exp.Eye Res. 63,661-672
28. Chen, Y.C., Reid G.E., Simpson, R.J. and Truscott, R.J.W. (1997) Molecular evidence for the involvement of alpha crystallin in the colouration/crosslinking of crystallins in age-related nuclear cataract. Exp.Eye Res. 65, 835-840

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ウ）著者名のあと1行あけて，著者名の，所属とその所在地（郵便番号付記）を記す．

エ）所在地のあと，4行あけて，本文を記す．

4．見出しは，区切りの大きいものから順に下記ア）～ウ）の通りとする．

ア）1，2，3，・・・

イ）1－1，1－2，・・・，2－1，2－2，・・・

ウ）a），b），c），・・・

各見出しのゴチ指定，改行等は既刊の実例にならう．

5．図，表，写真は所定の位置に貼る．図，表，写真の番号，表題，説明は和文原稿の場合にも英文で記すことが望ましく，そのまま写真製版出来るよう図，写真の下，表の上および下に記す．

6．和文原稿の場合には英文要旨をつける．

英文要旨冒頭には，表題，著者名，所属機関，その所在地（郵便番号付記）をこの順で記す．続いて，4行あけた後，ABSTRACT, KEY WORDS （10語以内）を記す．

7．英文要旨は英文原稿作成の手引きを参考にして記す．

8．英文要旨は表題から KEY WORDS まで含めて1頁以内に納める．

☆ 和文原稿作成の手引き

1．原稿は400字詰め原稿用紙に横書きで記す．ワープロを使用の場合は，25字×16行とする．

2．第1ページに和文表題，著者名，所属機関，その所在地（郵便番号を付記）を，この順序に記す．第2ページには英文要旨を記す．第3ページ以下に本文を記す．

3．見出しは，区切りの大きいものから順に下記ア）～ウ）の通りとする．各見出しのゴチ指定，改行等は，既刊の実例にならう．

ア）1，2，3，・・・

イ）1－1，1－2，・・・，2－1，2－1，・・・

ウ）a），b），c），・・・

4．図，写真および表は別紙とし，原稿中にはそれぞれの挿入箇所を指定する．

5．和文原稿の場合にも，図，写真および表の表題および説明文は英文で記すことが望ましい．

6．英文要旨冒頭には，表題，著者名，所属機関，その所在地（郵便番号を付記）を，この順序で記す．

7．英文要旨の後に KEY WORDS （10語以内）を記す．（日本語でのキーワードは不必要．）

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