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Profile of Gerontological Institutions

Twenty years of ageing research at the Mill Hill laboratories

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Abstract

Research on ageing was carried out in the Genetics Division laboratories, Mill Hill, London, from 1970 to 1990, resulting in more than 100 publications. The work centred around the in vitro ageing of human diploid fibroblasts, but there was also research on transformed cells, rat and mouse tissues, human lymphocytes, chick cells, mice and a microbial model system. The major conclusion from all this research, together with a broad overview of the whole field of gerontology, is that ageing has multiple causes, and that adult animals become senescent through the eventual failure of several important maintenance mechanisms. © 2002 Elsevier Science Inc. All rights reserved.

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1. Introduction: cellular ageing

In his article on 'UK research on the biology of aging' de Grey (2001) wrote that "only a few years ago, it could fairly be said that biogerontology research in the UK was in a sorry state". In contrast, I believe it can be proved that the Genetics Division, which was by far the largest group of investigators in UK, made a series of highly important contributions to the field during the period 1970–1990. de Grey cites only one of these contributions. I am reviewing here our most significant work.

In 1970, I was appointed by Peter Medawar, Director of the National Institute for Medical Research (NIMR), Mill Hill, London (Fig. 1), to set up a new Genetics Division with brand new facilities. I had been working mainly on genetic recombination and DNA repair in the Microbiology Division of the Institute, and this research would continue. I had also developed an interest in ageing, a field in which Medawar himself had made very important contributions. Hayflick (1965) had published a few years before his definitive study of the in vitro senescence of human fibroblasts, particularly strains WI-38 and WI-26, and at the nearby Institute of Biological Standards and Control (NIBSC) Jacobs et al. (1970) had characterised another human fibroblast strain, MRC-5. I decided to set up a research programme to study the possible mechanisms that determined the lifespan of these cells. MRC-5 cells have a lifespan of 50–70 population doublings (PDs) and it takes three to four months to complete a longevity experiment, starting with a frozen ampoule of early passage cells (usually passage 10–12). In our first experiments, we followed Hayflick's procedures, in which the cumulative split ratios in passaging the cells approximate to PDs. However, we soon used an electronic Coulter Counter to count the cells each time they were harvested, and in this way cumulative PDs were measured.

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Fig. 1. The National Institute for Medical Research, Mill Hill, London. The Genetics Division Laboratories are on the top floor of the central block.

It is well known that poikilothermic vertebrates have an extended lifespan at lower temperatures, and one of our earliest experiments was simply to incubate MRC-5 cells at 34, 37 and 40 °C, using cultures in quadruplicate (Thompson and Holliday, 1973). We demonstrated that the lifespan was no longer at 34 than at 37, but at 40 °C ageing appeared to be accelerated, because their senescence could not be reversed by transfer to 37. This seems to be the only study to show that temperature reduction has no effect on mammalian cell ageing. We also showed that senescent MRC-5 cells accumulate chromosome abnormalities (Thompson and Holliday, 1975), in agreement with the earlier work of Saksela and Moorhead (1963). Later, we studied the longevities of diploid and tetraploid cells and showed that they are not different (Thompson and Holliday, 1978). This provides strong evidence that recessive mutations are not an important cause of human fibroblast ageing. We soon realised that the ageing of cell lineages and cell populations are not the same, and this later lead to the formulation of the commitment theory of cellular ageing, which will be discussed later.

2. The stability of translation in protein synthesis

Orgel (1963, 1970) had suggested that the machinery for protein synthesis might be unstable, and that this could be a possible cause of ageing. Errors in the synthesis of proteins might feed back into the pathway of transcription and translation, thereby producing an ever increasing level of errors, and a so-called 'error catastrophe'. Several studies,

particularly by Gershon and Rothstein (reviewed by Rothstein, 1982), had shown that altered proteins often accumulate during ageing, and evidence for the instability of translation had been obtained in certain strains of fungi (Holliday, 1969; Lewis and Holliday, 1970). Leslie Orgel was an early year-long visitor to the Genetics Division and we had many discussions on theories of ageing, and how they could be tested. He subsequently wrote an important review on mammalian cell ageing (Orgel, 1973). We decided to search for a heat labile fraction of suitable 'housekeeping' enzymes, because it was known that random changes in the primary structure of a protein often produced less stable variants. Significant heat labile fractions of two enzymes (glucose-6-phophate dehydrogenase [G-6-PD] and 6-phosphogluconate dehydrogenase) were detected in senescent cells (Holliday and Tarrant, 1972).

At the same time, attempts were being made to measure the accuracy of protein synthesis in normal and senescent cells. In 1973, the distinguished Russian gerontologist, Zhores Medvedev, visited the Genetics Division for one year, but when he lost his citizenship he joined the scientific staff, and worked with his wife Margarita Medvedeva in the laboratory until his retirement. They demonstrated many significant changes in histones in rat and mouse tissues during ageing (reviewed by Medvedev and Medvedeva, 1991). Histone H1 does not contain methionine, so in principle it should be possible to measure the misincorporation of labelled methionine into purified fractions. In practice this is difficult to do, because small amounts of contaminating protein will contain labelled methionine. Nevertheless, experiments with both mouse tissue and human fibroblasts provided some limits for protein errors and showed that aging cells could not be replete with them (Buchanan and Stephens, 1978; Medvedev and Medvedeva, 1978). Until this day, the accuracy of protein synthesis in mammalian cells or tissues remains unknown, and most published experiments have provided only indirect information (reviewed by Holliday, 1996). It is known that abnormal protein molecules that arise during ageing are often due to post-synthetic changes such as oxidation, glycosylation, cross-linking, and so on, and such changes in enzymes could affect their specificity in a variety of contexts, including protein synthesis. Proteases can also preferentially remove abnormal molecules, and it was shown in the Genetics Division that senescent MRC-5 cells have an enhanced rate of protein turnover (Shakespeare and Buchanan, 1976).

A biometrician in the Institute, Tom Kirkwood, became interested in the theory of error propagation, and was also a post-graduate student in the Genetics Division. In a number of publications he defined the conditions under which error levels would be stable or might increase exponentially (reviewed by Kirkwood et al., 1984). Agents, which artificially increase error levels might shift cells from a stable to an unstable state. It was shown that the antibiotic paromomycin, which decreases the fidelity of eukaryotic ribosomes, also induces premature senescence in MRC-5 (Holliday and Rattan, 1984). Robert Rosenberger joined the staff of the Genetics Division, and he decided to explore the accuracy of transcription and translation in *Escherichia coli*, where much more powerful methods can be applied (reviewed by Rosenberger, 1991). He also obtained rather direct evidence that protein error propagation could be induced by streptomycin, with cell death occurring after about ten generations of growth (Rosenberger, 1982).

3. DNA replication and mutations

We were interested in DNA synthesis in fibroblasts, and in one of the earliest studies, Tom Petes, who was visiting the Microbiology Division, collaborated with us in measuring the rate of replicon elongation in young and senescent human fibroblasts. We showed that the rate was significantly reduced during ageing (Petes et al., 1974). Stuart Linn, an expert in DNA enzymology, came to work in the genetics Division for one year, and he measured the fidelity of DNA synthesis, using defined DNA templates, labelled triphosphonucleotides and cell free extracts. It was shown that fidelity was decreased using extracts from senescent cultures (Linn et al., 1976). Later, a graduate student, Vincent Murray, confirmed these results in greater detail, and also carried out a number of important control experiments (Murray and Holliday, 1981). Although it seemed very unlikely that mutation could be a primary cause of fibroblast ageing (Thompson and Holliday, 1978; Holliday and Kirkwood, 1981), it was nevertheless

possible that a general breakdown of cellular controls during ageing would result in the late induction of mutations, as was known to be the case for chromosome abnormalities. Evidence for this came from histological studies, which showed that cell variants increase with the expected kinetics, and by using other methods (Fulder and Holliday, 1975; Fulder, 1978).

Another visitor to the laboratory was Alec Morley, a haematologist from Adelaide, Australia. He wanted to measure somatic mutations in vivo, using cells other than fibroblasts. He harvested lymphocytes from donors of different age, and measured the frequency of those lacking HPRT (hypoxantine–guanine phosphoribosyl transferase) which are resistant to 6-thioguanine. For the first time, there was an unequivocal demonstration of an increase in bona fide mutations in somatic cells during ageing (Morley et al., 1982).

4. Limited and unlimited growth of human cells

Whereas normal human diploid cells have finite lifespan in culture, many cancer cells constitute permanent lines, which can grow indefinitely. Obviously it is very important to understand the change from the mortal to the immortal phenotype, and this may also throw light on the mechanism of cellular ageing. Cultured human fibroblasts have never been known to become immortalised spontaneously, in spite of the strong selection for this trait. In experiments with the oncogenic Simian virus (SV40), two permanent lines, MRC-5V1 and MRC-5V2, were isolated and characterised (Huschtscha and Holliday, 1983). In most cases, cells with a transformed phenotype entered a non-growing phase known as crisis, and then ceased growth. Immortalisation is also important when biochemical or genetic experiments cannot be done on a primary line with a genetic defect. This is true of Werner's syndrome, a recessive premature ageing disease. These cells also age prematurely in culture; have chromosome abnormalities and an increased level of heat labile G-6-PD (Holliday et al., 1974; Thompson and Holliday, 1983). An SV40 immortalised line of Werner's cells was also isolated (Huschtscha et al., 1986), and this has been used in other laboratories for genetic and biochemical studies. When these experiments were carried out, a biomarker of cell senescence was discovered. This was autofluorescence (AF) which can be quantitated in the FACS cell sorter. Normal MRC-5 cells have a low level of AF, but this increases exponentially throughout the in vitro lifespan (Rattan et al., 1982). Early passage Werner's syndrome cultures have a high level of AF, but its transformed immortalised derivative has a much lower level.

Another biomarker for in vitro ageing is the gradual loss of total methyl cytosine in DNA (Wilson and Jones, 1983), whereas immortalised cells retain a constant level. It is important to study the level of methylation in SV40 transformed MRC-5 cells before they entered crisis, and it was found that, unlike normal cells, they maintained a constant level of methylation (Matsumura et al., 1989). So this particular biomarker for ageing was removed even though the cells had finite lifespan. Interestingly, in more recent studies of DNA telomere length, it was found that both normal and pre-crisis cells have progressively shorter telomeres (Counter et al., 1992; Alsopp et al., 1992). The possibility that failure to maintain DNA methylation might eventually lead to cellular senescence received support from experiments with the potent demethylating agent 5-azacytidine using MRC-5 cells. Young cells treated once were unaffected, but their subsequent lifespan was greatly reduced, suggesting that they retained a memory of the demethylation event (Holliday, 1988; Fairweather et al., 1987).

Although many types of somatic cell have finite lifespan, germ cells are potentially immortal, and it was a reasonable supposition in 1970s that early embryonic cells can be immortal as well, as indeed has now proved to be the case. In a theoretical study, we proposed that cells become committed to senescence during development at a given frequency, and once a cell lineage becomes committed it has a defined finite lifespan before growth ceases (Kirkwood and Holliday, 1975). The commitment model or theory predicts that human fibroblast populations derived from early passage ampoules have a range of lifespan in PDs, which is well known, and also that population size is an important parameter in defining lifespan, which was subsequently confirmed by experiment (Holliday et al., 1977, 1981). Commitment could well be the loss of telomerase activity, or

the failure to maintain methylation, as discussed earlier.

Two other advances were made, one experimental and one theoretical. Hela cells were fused with Lesch Nyhan diploid fibroblasts and hybrids were selected. In almost all cases these hybrids had a finite lifespan, showing clearly for the first time that finite growth was dominant over the immortal phenotype (Bunn and Tarrant, 1980). Evidence in the literature indicated that senescent cells had a cell division block in G1 of the cell cycle, suggesting that there was a regulatory mechanism, but there were also good reasons to believe that there was a stochastic accumulation of molecular defects. To reconcile these observations Rosenberger et al. (1991) suggested that the cells responded to a given level of molecular defects by a stress response that blocked cell division irreversibly.

5. Development of the disposable soma theory of ageing

Three EMBO Workshops were held in 1970s and 1980s which discussed the accuracy of synthesis of DNA, RNA and proteins. Theories had been developed by Ninio (1975) and Hopfield (1974) that accuracy depends on proofreading and that proofreading steps consume energy. Also, the general principle emerged that speed of synthesis had to be balanced against accuracy of synthesis. In other words, in every biological situation there must be some optimum where there is a trade off between rate of macromolecular synthesis and accuracy. This may be different for germ cells and somatic cells, or in related species living in very different habitats. This general concept gave rise to the disposable soma theory of the evolution of ageing, which proposes that organisms have to divide available resources between the preservation of the soma, or body, and transmission of germ cells to the next generation (Kirkwood 1977; Kirkwood and Holliday, 1979). In this situation, there is a trade off between longevity and reproduction. Because natural populations are age-structured, it can be shown that Darwinian fitness is greater if the soma is not preserved indefinitely, and so becomes disposable. Initially, the theory considered mainly the investment of resources to maintain the integrity of macromolecules, which would most obviously include accuracy of synthesis, DNA repair and the removal of defective proteins. It later became apparent that this was too narrow a view, because maintenance of adult organisms also depends on the immune response, on the detoxification of harmful chemical in the diet, on the removal of oxygen free radicals, on wound healing, on epigenetic controls and on physiological homeostasis (Holliday, 1988). All these consume considerable energy resources, and are also controlled by many genes.

6. Conclusions

The research on ageing in the Genetics Division had its own evolution. Starting with studies on cells and proteins, it later included DNA synthesis and mutation, theoretical and experimental studies on the immortalisation of normal diploid cells, and the evolution of ageing. During the 20 years of research, about half the Division worked on ageing, and the other half on DNA enzymology, recombination and repair. For most of this period the total number of investigators was about 25, including scientific and technical staff, graduate students and visitors. Therefore at any time about 12 investigators were working on ageing, which made it the largest research group in the UK or Europe. The research on ageing attracted many visitors from abroad, with eight from USA, four from Australia, two from Russia, two from Greece and one each from Japan, India and Iran. The research is documented in more than 100 publications, and only a proportion of these are cited here. There was also work done on chick cells, with mice, and on the misincorporation of amino acids in proteins in bacteria, which could not be summarised here owing to the limitations of space. The research on ageing did not continue much beyond 1990. I left NIMR in 1988, two senior staff members, Zhores Medvedev and Robert Rosenberger, were soon due to retire, and Tom Kirkwood became professor of gerontology at Manchester.

Although the work started in 1970s with a fairly narrow approach, it ended with a broad one, which encompassed all aspects of ageing. Perhaps the main achievement of the Mill Hill school of ageing research was the realisation that many complex mechanisms

maintain the adult body, and that it is the eventual failure of these mechanisms that bring about senescence and death. This also means that ageing is multicausal, and that the 30-fold variation in the lifespan of different mammalian species is due to the different resources invested in maintenance mechanisms. I believe it can be said that at the end of the twentieth century that the biological reasons for ageing are finally understood, and I justify and elaborate this in my book *Understanding Ageing* (Holliday, 1995).

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