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Mechanisms of Carcinogenesis: Chemical Exposure and Molecular Changes

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THE NUMBER of proposed mechanisms of carcinogenesis is increasing, including indirect mechanisms such as cell proliferation [1–3]. This is of relevance to epidemiology for at least three reasons: (a) the identification of mechanisms of action of human carcinogens sheds light on the biological plausibility of a causal association between an exposure and cancer; (b) some of the most relevant findings on mechanisms have come from investigations in humans, i.e. potentially involving epidemiological expertise; (c) a complete understanding of the cancer process in humans is likely to arise from a combination of conventional epidemiological investigations, aimed at the identification of risk factors, and of biomarker-based studies, aimed at the elucidation of intermediate steps. In fact, cancer epidemiology has been practised until now mainly as a 'black-box' discipline, interested in the (presumed causal) association between an external exposure and the onset of a malignancy. Recently, however, the coupling of epidemiological design with the measurement of biological or biochemical endpoints such as DNA adducts, gene mutations or chromosome aberrations has started to contribute to the unravelling of the intermediate events occurring in the chain between the two extremes of exposure and disease.

Although several carcinogenic mechanisms have been proposed, we will review the epidemiological evidence concerning mutations in proto-oncogenes or tumour suppressor genes, i.e. genotoxic events. Genotoxicity is defined as the capacity of inducing a structural change of genetic material (point mutations; chromosome aberrations), and is particularly relevant to carcinogenesis when proto-oncogenes and tumour suppressor genes are involved. Proto-oncogenes are normal cellular genes that, when *activated* as oncogenes, cause alterations of growth and differentiation, thus enhancing the probability of neoplastic transformation. Tumour suppressor genes are normal cellular genes that, when *inactivated*, also cause alterations of growth and differentiation patterns [4].

Cell proliferation has also been proposed as a mechanism involved in carcinogenesis on the basis of two main considerations: (a) some non-genotoxic chemicals found to be carcinogenic in experimental animals also induce cell proliferation; and (b) an increase in the number of cells which have undergone a first change (e.g. proto-oncogene mutation) (clonal expansion of altered cells) increases the probability of a subsequent carcinogenic 'hit' in a multistep sequence [2]. Cell proliferation would thus increase the probability of cancer by making available a larger number of cells that are vulnerable to subsequent carcinogenic stimuli. Further developments in the theory of cell proliferation in carcinogenesis have invoked, for example, the role of peroxisome proliferation [5] or the accumulation of a particular protein in the rat kidneys [6].

The objective for this paper is to provide a critical review of a few studies involving the measurement of molecular endpoints pertaining to mechanisms of carcinogenesis, and to present an epidemiological point of view on the subject.

CHEMICAL EXPOSURE, GENE MUTATION AND CANCER

The involvement of proto-oncogenes and tumour suppressor genes in chemical carcinogenesis has been repeatedly proposed on the basis of different types of evidence: (a) transfection assays showed that mutated oncogenes were able to transform immortalised cells, i.e. to confer malignant properties; (b) chemical carcinogens are capable of producing mutations in specific loci of proto-oncogenes, for example, mutations in codons 12 and 61 of *ras* are induced by *N*-nitrosocompounds; (c) tumours induced in experimental animals with known carcinogens (*N*-nitrosocompounds, polycyclic aromatic hydrocarbons) showed a high frequency of mutated *ras* oncogenes. Also, epidemiological evidence has been recently published, suggesting the association between chemical exposure, oncogene mutation and cancer onset. The purpose of such studies is not to

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show that a certain exposure is carcinogenic, but to elucidate the relationship between exposure (usually an already known carcinogen), cancer risk and the supposed genotoxic mechanism. The target of such studies has important implications for their design.

A genotoxic mechanism putatively acting in chemically-induced human cancer is the description of a specific mutation of the p53 tumour suppressor gene in hepatocellular carcinomas from Qidong (China) and southern Africa [7–9]. These are areas of endemic contamination of food by aflatoxin B1, a known hepatocarcinogen. Among 16 patients in China, 8 had a transversion of the p53 gene; in southern Africa this proportion was 5 out of 10. The specificity of the mutation was double: it concerned a single site, the third base position of codon 249, and in most cases (11/13) was a G:C–T:A transversion, the same type of mutation known to be induced in DNA by aflatoxin B1 in experimental systems. From an epidemiological point of view, it would be of interest to have some additional evidence since: (a) the two studies are of the cross-sectional type, i.e. they have measured the p53 mutation in patients who had already developed cancer; (b) no information on individual exposure to aflatoxins is available. Regarding point (a), the observation which has been made is compatible with at least three interpretations: the p53 mutation lies within the causal chain leading from exposure to cancer; the mutation is a consequence of exposure but is not necessary to cancer development; the mutation is an epiphenomenon of cancer, as many genetic events take place in the cancer cell and relate to its 'genetic instability'. In other words, the temporal relationship (time lag occurring between exposure, p53 mutation and cancer onset) is still lacking in the observed association. As far as point (b) is concerned, knowledge of individual exposure is necessary to ascertain whether the mutation is present only in subjects exposed to aflatoxin B1 or also to other agents (as the hepatitis B virus, which is endemic in those areas). Thus, although the observation is suggestive, it is still waiting for a complete epidemiological cause–effect assessment, and its causal attribution entirely rests on the high specificity of the type of mutation.

Other investigations concerning p53 involved smoking and carcinoma of the head and neck [10], smoking and carcinoma of the lung [11], and radon-associated lung cancer [12]. Field *et al.* [10] examined the expression of the p53 gene, using two different antibodies, among 73 patients with cancer of the head and neck. They found overexpression in 67%. Actually, only one out of seven non-smokers showed overexpression, vs. 29 out of 37 smokers [odds ratio = 22, confidence interval (C.I.) 3.5–135]. Also, of a group of 10 patients who had given up smoking more than 5 years before, 9 had elevated expression of p53. The type of test used, however, does not allow the inference of whether a specific p53 mutational spectrum was present in smoking patients as opposed to non-smokers or other cancer patients.

Suzuki and colleagues examined 30 non-small cell carcinomas of the lung, and found p53 mutations in 14 [11]. The mutations were mainly of the G to T type, and were closely associated with smoking habits, with an estimated odds ratio of 5.3 for smokers of 20 cigarettes per day or more, compared to non-smokers.

Vahakangas and colleagues have investigated p53 and *ras* mutations in 19 uranium miners exposed to radon, affected by lung cancer [12]. Among the 19 subjects, 7 had p53 mutations. None of these were G:C to T:A transversions, i.e. the mutation types which have been observed more frequently in clinical series of lung cancer patients who smoked. Two of the mutations were deletions, which have been reported only rarely in lung cancer. Mutations clustered between codons 146–161 and 195–208, an observation judged to be unusual in lung cancer.

Investigations concerning the *ras* oncogenes have mainly considered the association between lung cancer and tobacco smoking. K-*ras* mutations have been demonstrated as a feature of non-small cell lung carcinoma, and have been found to be associated with heavy smoking. In a study on 27 smokers and 27 non-smokers affected by lung adenocarcinoma, K-*ras* mutations were found in eight smokers and two non-smokers (odds ratio = 5.3, 95% C.I. 1.1–25) [13]. All mutations were in codon 12, mostly G to T transversions, as in other investigations on lung cancer [14]. G to T transversions have also been shown in lung tumours induced in mice with benzopyrene [15]. In a second study on 48 lung cancer patients, K-*ras* mutations were found in 14 specimens (and in 12 out of 21 adenocarcinomas) [16]. Also in this case, the commonest types of mutations were G to T transversions in codon 12. A clear association with heavy smoking was found (odds ratio = 4.9, 90% C.I. 1.2–19.5), and also with occupational asbestos exposure (odds ratio = 2.2, 90% C.I. 0.6–8.7). However, all the patients heavily exposed to asbestos were also current or former smokers.

DISCUSSION

Issues of study design

The mentioned investigations used a cross-sectional approach to answer mechanistic questions, i.e. whether among a group of cancer patients those exposed to a specific chemical showed more frequently genotoxic manifestations (gene mutations) than unexposed patients. This type of approach raises some methodological issues: (a) after the identification of a homogeneous series of cancer patients, exposure assessment, collection and storage of biological material and the ensuing analysis of mutational spectra should be strictly blind; (b) exposure assessment should be extremely accurate, since it is hypothesised that a specific mutation, such as a G:C to T:A transversion at a particular codon, is caused by a specific chemical; (c) covariates which could act as potential confounding factors should be accurately identified.

Concerning point (a), it has been shown that polymerase chain reaction (PCR) analysis is open to false positive results: this is due to contamination, resulting from 'carry-over' from previous PCR reactions [17]. In fact, in performing PCR it is advisable to use separate rooms for initial processing of specimens, setting up of the assays, running the amplifications, and analysing the PCR products [17]. If these procedures are not used, and the analysis is not blind, i.e. the exposed and unexposed cases are examined at different times, spurious association with exposure can be found. Also, the use of 'historical controls' is not advisable.

As far as point (b) is concerned, an example about chromosomal aberrations, another well-known genotoxic carcinogenic mechanism, will serve. Alterations which are frequent in some forms of human cancer are the Philadelphia chromosome in myeloid leukaemia and the 8–14 translocation in Burkitt's lymphoma. It has been proposed that the latter translocation is caused by the putative agent of Burkitt's lymphoma [the Epstein–Barr (EB) virus] [18]. With this possible exception, there is no other case of a clear association between an external exposure, a specific chromosome aberration and human cancer. Several attempts have been made to demonstrate an association between chemical exposures, chromosome aberrations and malignancies, particularly in adult leukaemias. Unfortunately, in such investigations the weakest part was often represented by

the assessment of chemical exposure, being based on generic classifications such as 'chemical agents' or 'mutagens'. When the results of these studies are considered over time, it is obvious that the relative risks (i.e. the number of times that some chromosome aberration appeared more frequently in leukaemia patients exposed to chemical agents, compared to those not exposed) dropped from around 15 in the first investigation to around 1 in the most recent ones [19]. This drop is very likely due to increasing accuracy of exposure assessment procedures, once again indicating the need for a thorough consideration of the epidemiological methods when evaluating data concerning carcinogenic mechanisms in humans.

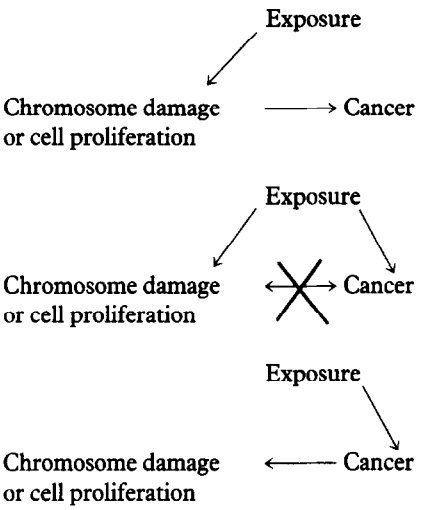
Concern about confounding factors should always be expressed in studies of molecular epidemiology. For example, a typical confounding phenomenon might arise from the fact that exposed workers have a lower social class and more advanced stages of cancer at diagnosis: therefore, they could show overexpression of some genes which are associated with the clinical stage, rather than with the risk of disease. Another example is a study which found overexpression of the p55 protein in patients with transitional cell carcinoma of the bladder compared to control subjects. The authors of the study did not report descriptive information on cancer patients' prior therapeutic treatment. Since half of the cancer patients had advanced disease, presumably they had been treated with radiation and/or chemotherapy, which are mutagenic and might have induced in themselves the observed altered gene expression (example cited in [20]).

Relevance to the causal chain and degree of evidence

We have already considered the uncertainties involved in the interpretation of the temporal sequence of exposure to aflatoxins, p53 mutation and liver cancer onset. Also in the case of another putative mechanism of carcinogenesis, cell proliferation, similar concerns can be raised. It is certainly possible that cell proliferation plays some role in carcinogenesis, and the idea that a larger number of cells are made available for subsequent carcinogenic stimuli is attractive. However, the observation that cell proliferation accompanies carcinogenesis is compatible with at least three alternative explanations (in addition to the 'causal' one): (a) enhanced proliferation is just a *property* of cancer or precancerous cells; (b) proliferation may be the *side-effect* of different chemical treatments, including carcinogenic compounds; (c) proliferation may be the *epiphenomenon* of some damage induced by carcinogens. In all three cases proliferation is not necessary to cancer onset, i.e. it does not belong to the causal chain between exposure and the tumour. Crucial experiments and epidemiological observations can be conceived in order to ascertain the role actually played by cell proliferation. For example, it has been claimed that some chemicals, including D-lemonene, gasoline and tetrachloroethylene, cause kidney cancer through cell proliferation due to the accumulation of a particular protein (alpha-2μ-globulin) in the kidneys of male rats. This mechanism would be active only in male rats and not in other species, and this would thus hamper the ability to extrapolate the results of tests from animals to humans. However, at least two questions can be raised concerning the experimental basis of this reasoning: (a) is there any evidence that the same chemicals do not cause cancer in species which do not accumulate alpha-2μ-globulin? (b) if one blocks the accumulation of the protein by the means of other chemicals, do kidney tumours develop in male rats?

Epidemiological studies provide some evidence that the kidney might be the human target organ of the carcinogenic properties of at least two of the chemicals mentioned, gasoline and tetrachloroethylene, although there is no evidence of alpha-2μ-globulin accumulation.

There are several distinct aspects which need to be addressed when evaluating the evidence that a certain mechanism is active in cancer induction. One is its relevance, i.e. the evidence that it belongs to the causal chain. The following scheme represents three different possible interpretations of an empirical observation:



Evidence about the appropriate time sequence is obviously needed before concluding in favour of the mechanistic role of a particular process or its associated biomarker.

In addition, mechanistic observations are often made in test systems which are not the same as those to which we extrapolate the finding (e.g. target cells different from those of the cancer type of interest; animal species different from humans; etc.).

As far as the 'degree of evidence' of the mechanistic involvement of a certain observation is concerned, criteria can be developed, analogous to those introduced by Sir Bradford Hill for epidemiological associations:

(1) Is the method used to investigate the mechanistic role of an endpoint reliable and reproducible?
(2) Have the agent and the endpoint been studied in different species and/or different test systems?
(3) Are there potential alternatives to a cause–effect interpretation of the findings (including bias, confounding or chance)?
(4) Is the design of the study which disclosed the association appropriate and meaningful?
(5) Is the association between the agent, the measured endpoint and cancer compatible with background knowledge on the carcinogenic process?

The analogy with criteria developed for the interpretation of traditional epidemiological studies is quite justified, since also the mechanistic evidence is likely to be of the stochastic type. In fact, even for the strongest examples of a mechanistic role of a certain biomarker, exceptions to a rigidly deterministic association exist: not all chronic myeloid leukaemias are positive for the Philadelphia chromosome, nor do all Burkitt lymphomas show the typical 8–14 translocation. Therefore, considering the importance that mechanisms are assuming in the interpretation of epidemiological and experimental findings of carcinogenesis,

to develop sound criteria for the evaluation of evidence would be important.

Finally, the development of such criteria serves to underscore the growing interdependence of both traditional and molecular epidemiological approaches in cancer epidemiology. Investigations of carcinogenic mechanisms in human populations based on molecular events has been viewed by some as representing a significant philosophical shift in epidemiological research that threatens to topple traditional epidemiological paradigms [21]. However, we hope the present discussion serves to demonstrate that quite the opposite is true. Mechanistic studies will only truly enhance understanding of human carcinogenesis not when they replace traditional epidemiology, but, as indicated by the 'degree of evidence' schema above, when they are successfully integrated into traditional epidemiological thinking.

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