Comparison of the significance of three histopathological thresholds of malignancy in experimental colorectal tumours

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SUMMARY The purpose of this experiment was to study sequential histogenesis of colonic epithelial tumours in the dimethylhydrazine (DMH) induced rat colon cancer model. Seventy outbred female Wistar rats treated with DMH (40 mg/kg, subcutaneously weekly for five weeks) were killed and autopsied in batches of 10 every five weeks from the 10th to 40th weeks from first treatment. The resulting 378 colonic lesions were assigned to benign or malignant categories using each of three standard histopathological thresholds of malignancy: α, the transition from dysplasia to intra-epithelial carcinoma; β, invasion through the crypt basement membrane; and γ, invasion through the muscularis mucosae. These comprised 79 'benign' and 299 'malignant' or 273 'benign' and 141 'malignant' lesions depending on the threshold (α or γ) assigned (p<0.001). Decreasing ratios of pre-threshold to post-threshold lesions between 15 and 40 weeks (α, 2.0 to 0.051; β, 3.5 to 0.57; γ, 8.0 to 0.87) provide some support for an 'adenoma-carcinoma' progression for each. Comparison of time-dependent prevalence curves confirms that the α threshold (cyto-architecture) is qualitatively different from the β and γ thresholds (invasion), showing that the adenoma-carcinoma and de novo hypotheses need not be mutually exclusive. The time-dependent prevalence data support de novo origin of some carcinomas, as well as at least two other modes of accrual for neoplastic lesions.

Colorectal carcinoma (CRC) now ranks second only to lung cancer as a cause of death from cancer in the Western world. By definition, this tumour arises from colorectal epithelium but the nature of its histological precursor(s) has been controversial. Some workers believed that CRC arises de novo in previously normal epithelium, while others argued for an origin in a pre-existing benign colonic neoplasm, the adenomatous polyp. The distinction between these two hypotheses remains of critical importance in understanding the pathogenesis of the disease, because of their different aetiological and preventive implications. The former hypothesis favours a direct, one step transformation of normal into cancerous cells, while the latter favours a more complex, multistage phenomenon.

The proponents of the adenoma-carcinoma sequence adduced a comprehensive body of epidemiological, clinical, and histopathological evidence to support the idea that most human colorectal carcinomas arise from adenomatous polyps. Final pathological proof is difficult to obtain in man, however, as most adenomatous polyps are surgically removed when discovered, thereby interrupting the natural course of the disease. For this reason, attention turned to the use of animal models of colon cancer, where colonic tumours can be studied under controlled laboratory conditions.

Although some experimentalists could claim evidence for an adenoma-carcinoma sequence, they mainly supported a de novo origin for the majority of chemically induced colon carcinomas. It is now becoming appreciated that in appropriate conditions either hypothesis for the route to invasive cancer may apply in man as well as experimental animals.
The aim of the present experiment was to follow the histogenesis of colonic epithelial tumours in groups of rats treated with a short course of dimethylhydrazine, a colon carcinogen, and subsequently killed at standard intervals. By using three different histopathological thresholds in classifying the same set of tumours, we were able to compare progression through the different thresholds over time. It was anticipated that this approach would refute or support the general concept of an adenoma-carcinoma sequence.

Methods

ANIMALS
Seventy weanling outbred female Wistar rats (Tuck & Sons, Battlesbridge, Essex, UK) weighing 50-80 g were weaned onto a standard pelleted laboratory diet (MRC Formula 41B, Dixon & Sons, Ware, Herts, UK) and water, both given ad libitum. They remained on this diet for the duration of the experiment. Outbred rats were chosen to simulate the genetic heterogeneity of man.

CARCINOGEN TREATMENT
1,2-dimethylhydrazine dihydrochloride (Aldrich Chemical Co, Gillingham, Dorset, UK) was prepared according to the method of Filipé.11 Each rat received a colon cancer producing regimen of DMH injections (40 mg/kg body weight, sc weekly for five weeks) in the left flank. The rats were housed in temperature controlled quarters in subgroups of five in suspended cages with open mesh, wire floors designed to prevent coprophagia. They were weighed weekly and inspected daily for signs of illness.

AUTOPSIES AND HISTOPATHOLOGICAL PROCESSING
Ten weeks after the first DMH injection and at five weekly intervals until the 40th week, random groups of 10 rats were anaesthetised, killed, and autopsied. All macroscopically abnormal colonic tissue was sampled, fixed in 10% formaldehyde, routinely processed and embedded in paraffin wax. Sections cut from the paraffin blocks were stained routinely with haematoxylin and eosin and examined and described independently by two pathologists.

HISTOPATHOLOGICAL EVALUATION
Histopathologists observe a continuous spectrum of neoplastic changes in the colon and rectum which is difficult to divide into subsets.21 Nevertheless they must allocate individual lesions into categories with prognostic value. To do this, they describe the architectural and cytological features of such lesions, and, using agreed histological thresholds of malignancy, separate prethreshold (benign) from postthreshold (malignant) lesions. The threshold chosen may vary according to the purpose for which it is intended – that is, whether clinical or scientific.

Some pathologists consider that intramucosal and intraepithelial carcinoma are self-contained when surgically excised in a polyp, and, in consequence, are of no further clinical significance to the patient.24 To avoid confusion in management, they prefer to report polyps which contain intraepithelial or intramucosal carcinoma as ‘adenoma with severe dysplasia’ or ‘adenoma with severe atypia’.25 Epidemiological records using these operational terms, however, could be retrospectively misconstrued.

To avoid any inconsistency in the choice of histopathological terms, a new systematic approach, which recognises the morphological spectrum of colonic epithelial neoplasia and also allows for simplicity in analysis, is adopted in this study. We propose that this spectrum be divided into four subsets of increasing malignancy separated by three thresholds, called α, β, and γ respectively.

α defines the thresholds between intra-epithelial dysplasia of moderate severity and carcinoma in situ (CIS),

β defines the threshold between CIS and intramucosal carcinoma – that is, invasion into the lamina propria, and

γ defines the threshold between intramucosal and transmucosal carcinoma – that is, invasion beyond the muscularis mucosae into the submucosa.

Terms such as pre-α or post-β accurately specify all the lesions to one or other side of any chosen threshold, without resorting to the terms benign or malignant. The economy of this terminology emphasises that the only property described is the position that the lesion occupied at the time it was sampled in the continuous spectrum of development of neoplasia in the organ. Every lesion can be classified in relation to all three thresholds, allowing the comparisons described below.

DEFINITION OF HISTOPATHOLOGICAL SUBSETS BETWEEN THRESHOLDS
To avoid any ambiguity and to provide easy reference, we used the following well recognised histopathological definitions for the four subsets lying between the thresholds.

Dysplasia
An unequivocal neoplastic epithelial proliferation characterised by nuclear stratification, pleomorphism, hyperchromatism, and loss of polarity,26 but
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excluding the most severe degrees described as carcinoma \textit{in situ} (below).

\textit{Threshold} $\alpha$

\textit{Carcinoma in situ (CIS), (Intra-epithelial carcinoma)}

A neoplastic epithelial alteration, equivalent to the severest degrees of dysplasia, in which abnormal epithelium is confined to the original contours of the crypt of Lieberkühn without invasion of the intervening lamina propria.\textsuperscript{25,26}

\textit{Threshold} $\beta$

\textit{Intramucosal carcinoma}

Invasion of the lamina propria by cytologically malignant epithelium or glands without extension through the muscularis mucosae.\textsuperscript{31,36}

\textit{Threshold} $\gamma$

\textit{Invasive carcinoma}

Cytologically malignant epithelium or glands which invade beyond the muscularis mucosae into the submucosa or beyond.\textsuperscript{25,26}

Figures 1 to 5 illustrate examples of these lesions. In this study we also examined the ratios of the prevalence of pre- and post-threshold lesions for each threshold for each five week period. We expected that the series of ratios for each threshold would show the relationship over time between the pre-threshold (‘adenoma’) and post-threshold (‘carcinoma’) lesions for that threshold. Theoretically, if there is any direct evolutionary relationship between the ‘adenoma’ and ‘carcinoma’ lesions, this will be reflected in changes in the ratio over time. If, however, the two tumour types are unrelated, the numbers of pre- and post-lesions will change independently. In this way, we were able to compare the reliability of the three histopathological thresholds of malignancy as indicators of the adenoma-carcinoma sequence in experimental colorectal tumours.

Where appropriate, results were analysed statistically by the $\chi^2$ test.

\textbf{Results}

Forty weeks after the start of the experiment, a total of 378 colonic tumours had been diagnosed in the 70 rats autopsied. The total numbers of tumours of each
histological type in each autopsy batch (of 10 animals) are given in Table 1. The position of each of the three thresholds, α, β and γ, is also shown in Table 1. The ratios of the number of lesions which lie to either side of each threshold (its adenoma-carcinoma ratio) are given for each autopsy batch in Table 2. Comparison of the prevalence of pre- and post-threshold lesions in each autopsy batch—that is, at each time period—are illustrated in Figure 6.

(1) Inspection of the data in Table 1 shows a progressive trend to increased numbers of histological lesions with time in every category except pre-α (dysplasia).

(2) The rise and subsequent fall of the pre-α lesions (Fig. 6, threshold α) shows that these lesions with only mild to moderate dysplasia accumulate and most are subsequently lost, probably to post-threshold forms.

(3) The accumulation of post-α lesions with time (Fig. 6, threshold α) suggests that the post-α lesions accumulate at the expense of pre-α lesions. The late increase in post-α forms, however, far exceeds the number available from the pre-α group and suggests that this stage may be passed very rapidly or omitted.

(4) The pre-β and pre-γ curves (Fig. 6, thresholds β and γ) both show initial accumulation and then some loss followed finally by apparently exponential accumulation. The two rates of accrual reveal two biological processes. Of the initial burst of lesions, some and possibly all are lost (presumably) to the more severe post-threshold stage. This effect is, however, subsequently overtaken by an exponential form of accrual and some (possibly all) of these progress to the post-threshold stage.

(5) The exponential trend in the post-γ curve confirms previous work showing that invasive adenocarcinoma induced in rat colon accrues in this way.

(6) The occurrence of one of the few mucous/signet ring cell carcinomas as a post-γ lesion earlier than the first pre-α lesion (Table 1) shows that not all malignant lesions are preceded by a benign one. Furthermore, Table 1 confirms that this histopathological entity occurs at a low steady rate over time which contrasts strikingly with the accrual of the majority of lesions described in paragraph 4 above.

(7) Comparison of the numerical effects of using the different histological thresholds to analyse this population of tumours produces interesting results. For example the succession of ‘adenoma-carcinoma’
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(pre-threshold/post-threshold) ratios for each threshold (Table 2) show a trend, decreasing over time, for all three. This provides experimental support for the concept that pre-threshold (‘benign’) lesions progress to post-threshold (‘malignant’) lesions over time in the majority of cases. At a given point in time, however, use of different thresholds produces significantly different numbers of lesions in each category, and the quantitative effect can be considerable (Table 1). Taking the whole population of lesions and using threshold $\alpha$ as the criterion of malignancy, we obtain 79 ‘adenomas’ (pre-$\alpha$ lesions) and 299 ‘carcinomas’ (post-$\alpha$ lesions). Similarly use of threshold $\gamma$ produces 237 ‘adenomas’ (pre-$\gamma$ lesions) and only 141 ‘carcinomas’ (post-$\gamma$ lesions). This is a statistically significant difference ($p<0.001$).

Discussion

Madara et al. indicate a reason for the failure of the proponents of the adenoma-carcinoma and de novo hypotheses to agree. They argue that early morphological descriptions in experimental animals13–14 predate the morphological criteria that have been widely used to describe the adenoma-carcinoma sequence in man. Reference to these criteria reveals the contradiction between cytological and invasive thresholds for malignancy. The cytological appearances, which were regarded as ‘fundamentally the same’ in all types of benign tumour (adenoma), were divided into the same three levels of atypia (mild, moderate, and severe) as are generally accepted as precancerous dysplasia in ulcerative colitis.3 They also specified that: ‘Severe atypia includes those features sometimes named focal carcinoma or carcinoma in situ without invasion across the line of the muscularis mucosae….’. They therefore include as benign some tumours with cytological appearances which are considered to be malignant, on the grounds that a threshold used as a starting line for invasive carcinoma has not yet been crossed. When Madara et al. applied these criteria rigorously in the DMH-rat model an adenoma-carcinoma sequence could be described. They concluded that the failure of other experimentalists to interpret similar lesions in the same way is due to the use of different diagnostic criteria.

To avoid such problems, the histopathological
spectrum of colonic lesions in this experiment was divided into four subsets of increasing malignancy, and the three thresholds between them called \( \alpha \), \( \beta \) and \( \gamma \). By describing lesions by their relation to these thresholds, pre-\( \alpha \), post-\( \beta \), etc, we could define their position in the spectrum avoiding terms such as adenoma and carcinoma, which also have prognostic implications. However this assumption does imply that the spectrum represents a unique succession of stages.

Table 1  Histopathological classification of the colorectal lesions in each batch of 10 rats autopsied at five week intervals

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Dysplasia</th>
<th>Carcinoma-in-situ</th>
<th>Intramucosal carcinoma</th>
<th>Invasive Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (1)* 1</td>
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<tr>
<td>15</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1 (1) 9</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>2</td>
<td>3</td>
<td>15 (1) 47</td>
</tr>
<tr>
<td>25</td>
<td>18</td>
<td>6</td>
<td>4</td>
<td>53</td>
</tr>
<tr>
<td>30</td>
<td>18</td>
<td>7</td>
<td>4</td>
<td>24 (2)</td>
</tr>
<tr>
<td>35</td>
<td>20</td>
<td>11</td>
<td>11</td>
<td>58</td>
</tr>
<tr>
<td>40</td>
<td>8</td>
<td>52</td>
<td>17</td>
<td>166</td>
</tr>
<tr>
<td>Total</td>
<td>79</td>
<td>116</td>
<td>42</td>
<td>378</td>
</tr>
</tbody>
</table>

*() indicates the number of invasive signet ring cell or other mucinous adenocarcinomas included.

The results show a clear accumulation of post-threshold lesions over time at the expense of pre-threshold lesions whichever threshold is used. This is illustrated by the changes in the ratios of pre- to post-threshold lesions (Table 2). As the ratios for each threshold tend to decline progressively, this provides some support for the hypothesis that there is a direct evolutionary relationship between the pre- and post-threshold lesions, and so for an adenoma-carcinoma sequence. The \( \alpha \) threshold and \( \beta \) and \( \gamma \) thresholds, however, are qualitatively different (Fig. 6), as tumour behaviour is assessed by cyto-architecture.

Table 2  Ratios of the prevalence of pre- and post-threshold lesions for each threshold at each time

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Thresholds</th>
<th>( \alpha )</th>
<th>( \beta )</th>
<th>( \gamma )</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>2-0</td>
<td>3-5</td>
<td>8-0</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0-57</td>
<td>2-92</td>
<td>4-22</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0-69</td>
<td>3-5</td>
<td>21-0</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0-51</td>
<td>0-89</td>
<td>1-21</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0-26</td>
<td>1-23</td>
<td>2-87</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0-051</td>
<td>0-57</td>
<td>0-87</td>
<td></td>
</tr>
</tbody>
</table>
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Fig. 6  Comparison of the prevalence of pre- and post-threshold lesions at each time period using the three thresholds of malignancy discussed in the text.

and invasion respectively. We conclude that the de novo and adenoma-carcinoma hypotheses, as they use qualitatively different criteria, are not mutually incompatible.

The accrual patterns of pre-β and pre-γ lesions (Fig. 6) indicate that there are at least two main biological processes. It is also clear from the data (Table 1) that not all malignant (post-threshold) lesions are preceded by a benign one, as some malignant tumour phenotypes such as the signet ring cell carcinoma effectively omit a prethreshold stage, arising de novo as post-α lesions. These three accrual patterns confirm other reports.22–25 Therefore although the process must be a linear anatomical progression, the implication of a single qualitative spectrum is too simple.

Salomon24 has discussed how the World Health Organisation (WHO) and International Union against Cancer (UICC)/American Joint Committee on Cancer classification systems imply that cancer is a single linear process. Difficulty in distinguishing some adenomatous proliferation from well differentiated intramucosal carcinoma has accentuated the problem of diagnosis in the colon. The WHO24–25 use the word carcinoma only when a colonic neoplasm shows invasion by tumour tissue across the muscularis mucosae. The 'TNM' system of the UICC25 relies for the diagnosis of malignancy on architectural and cytological criteria which can be independent of this threshold of invasion. In general carcinoma in situ (Tis) is listed without qualification, although 'an intraepithelial tumour without invasion of the lamina propria' is specified for stomach. In the current edition intramucosal carcinoma has been deleted from the TI category in colon and rectum,18–30 and by default must now be recorded in the carcinoma in situ category (Tis).

When using the anatomical thresholds in the histopathological assessment of malignancy, their limitations should be appreciated. The α-threshold is the most sensitive, as carcinoma is defined, while still in situ, on architectural and cytological criteria alone with no invasion specified. This would overestimate the numbers of biologically malignant carcinomas as not all the lesions so described need progress. While this may be a suitable endpoint for an experimentalist, it could cause unnecessary interventions in human disease. α might also be a suitable threshold in epidemiological studies of early colonic cancer.

β is conceptually the most important as it defines
the threshold beyond which a lesion could be considered invasive. By breaching the crypt outline and invading the mucosa, the cells in theory have access to the vasculature and therefore may metastasise. In this study, intramusoccal carcinomas (lesions between $\beta$ and $\gamma$ thresholds) were the least common type of tumour encountered. This could be caused by rapid progress through this stage or difficulty in assigning lesions to this category, even by experienced observers.

$\gamma$ is the threshold where breaching of the muscularis mucosae must have occurred. It is used by many surgical pathologists largely because it is considered to be the best operational definition of clinical cancer of the colon, and is easy to apply. The use of this threshold to diagnose malignancy in an experimental situation, however, as in the present study, produces significantly smaller numbers of carcinomas. By lumping all the pre-$\gamma$ lesions into the ‘adenoma’ category, use of this $\gamma$ threshold could result in the loss of data in, for example, human epidemiological studies or prognostic follow up studies.

The widespread introduction of colonoscopes for tumour diagnosis clinically and also experimentally has changed the quantity and anatomical extent of the material available for pathological evaluation. The absence of the muscularis mucosae in the polypectomy specimen may make it impossible to assess the $\gamma$ threshold. Resultant allocation of the lesions to the pre-$\gamma$ category means that data will be lost or excluded from the study.

The different histopathological thresholds of malignancy used in this study are all useful in different experimental and clinical contexts. Experimentalists and surgical pathologists are encouraged to define the thresholds chosen and their reasons for using given thresholds in future publications. This could obviate misunderstandings and inconsistencies which have characterised the past debate about the histogenesis of colonic cancer.

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