Aggregated Colon Cancer Cells Have a Higher Metastatic Efficiency in the Liver Compared with Nonaggregated Cells: An Experimental Study

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Submitted for publication September 16, 2002

INTRODUCTION

Metastasis to the liver is the commonest way of spreading in patients with colorectal cancer, and almost all patients with liver metastases will die of their disease [1]. Nevertheless, the development of metastasis is a highly selective process in which the metastatic efficiency is determined by the least-efficient step. Therefore, the overall efficiency of the whole metastatic process itself is low. It has been demonstrated experimentally that, after injection of metastatic cancer cells into the mesenteric vein, only 0.68% of these cells form liver metastases [2]. In contrast with viable single or nonaggregated cancer cells that often fail to form metastases, cancer cell clumps or aggregates result in a high metastatic efficiency after injection via the portal vein [3]. Although the absolute number of cancer cells required for metastasis in humans is unknown, a positive relationship between the number of circulating cancer cells and the subsequent development of metastases has been the subject of several experimental studies [4–7]. It is also demonstrated in animal models that both single nonaggregated and aggregated cancer cells are shed into the vascular drainage system of the primary tumor [6, 8]. However, the metastatic efficiency of nonaggregated single cancer cells compared with cancer cell aggregates remains unclear for equivalent numbers of cancer cells.

The predilection for colorectal cancer to metastasize to the liver is related partly to the fact that the gastrointestinal tract is drained by the portal vein from where tumor emboli may arrive via the mesenteric veins. The majority of these circulating cancer cells survive the mechanical trauma and host defense mechanisms encountered during their passage through the vascular system, but most of these cells die or become “dormant” once lodged within the hepatic parenchyma.

Background. It remains unclear whether aggregated colon cancer cells have a higher tendency for metastasis formation than nonaggregated cells. Also, the absolute number of cancer cells required for hepatic metastasis remains undefined. The aim of the present study was to compare in the liver the metastatic efficiency of viable nonaggregated colon cancer cells versus cell aggregates for equivalent numbers of cancer cells.

Materials and methods. DHD/K12/TRb colon cancer cells were administered through the portal vein in syngeneic male BD IX rats. Surgical exploration was performed 8 weeks after injection. Four groups of rats were injected with 0.25 or 0.5 \( \times 10^6 \) DHD/K12/TRb viable cancer cells, either as single nonaggregated cells or as cell aggregates.

Results. Hepatic metastases were observed in 81% of the rats after intraportal injection of cell aggregates equivalent to 0.5 \( \times 10^6 \) cancer cells. A significant lower metastatic efficiency was found after the injection of 0.5 \( \times 10^6 \) non-aggregated, and 0.25 \( \times 10^6 \) aggregated or nonaggregated cancer cells i.e., 16%, 32%, and 27%, respectively.

Conclusion. Aggregated colon cancer cells have a higher metastatic efficiency in the liver compared with non-aggregated cells, although a critical number of cancer cells are necessary. © 2003 Elsevier Inc. All rights reserved.

Key Words: liver metastases; colorectal cancer; animal.

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This hypothesis of dormant tumor cells or micrometastases in the liver has been supported only by experimental evidence: undetectable liver metastases could be stimulated into growth by (repeated) surgical trauma or by immunosuppression with Cyclosporin-A [3, 9].

In the present study DHD/K12/TRb colon cancer cells were administered through the portal vein in syngeneic male BD IX rats. The objectives were to 1) compare the metastatic efficiency and localize cancer cells in the liver after intraportal injection of viable single nonaggregated cancer cells versus cell aggregates and 2) evaluate the effect of immunosuppression with cyclosporin A on tumor growth in rats with hepatic “micrometastases” or “dormant” cancer cells.

MATERIALS AND METHODS

Animals

Syngeneic male BD IX rats (12–14 weeks old) were purchased from Harlan United Kingdom Limited (Shaw’s Farm, Blackthorn, Bicester, Oxon, OX6 OTP, UK). The animals were given free access to standard laboratory chow and water throughout the experiment. Surgical exploration under ether-anesthesia was performed at 8 weeks after cancer cell injection to evaluate metastatic efficiency. The experimental protocol was approved by the local institutional review committee, and meets the guidelines of the national governmental agency.

Cell Culture

The DHD/K12/TRb cell line was obtained from the European Collection of Animal Cell Cultures (ECACC). The DHD/K12 cell line is an established transplantable carcinoma cell line originating from a 1,2-dimethylhydralazine-induced colon adenocarcinoma in BD IX rats [10, 11]. The subline DHD/K12/TRb (PRob) gives progressive tumors in syngeneic rats in which it is inoculated, whereas the subline DHD/K12/TBs (REGb) also gives tumors in syngeneic rats but these tumors disappear within 3 or 4 weeks [12, 13].

DHD/K12/TRb colon cancer cells were grown in monolayer culture with medium containing DMEM + Hams F10 (1:1), 2 mM l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum under standard conditions. Cells were trypsinized once and washed in PBS twice, to completely desaggregate the cells for either injection of single cancer cells or formation of cell aggregates.

Cell Count

The number of viable and nonviable cells was counted in a haemocytometer (200× magnification) after trypan blue staining. Cancer cell suspensions were prepared to obtain 0.25 × 10⁶ or 0.5 × 10⁶ viable single cells per 0.5 ml suspension for intraportal injection in group A or B, respectively.

Cell aggregates were formed, with the same medium as used in monolayer culture, in RCCS-D vessels (Rotary Cell Culture System Disposable) in 5% CO₂ incubator at 37°C, at a circling velocity of 30 rpm during a period of 10–14 days. Six RCCS-Ds were examined independently for cell count. The content (50 ml) of RCCS-Ds was divided into two equal volumes. The first volume was used for intraportal injection and the second volume for validation of the number of viable cells. The first volume was centrifuged at 1200 rpm for 5 min, and the pellet was suspended in 5 ml of medium. Subsequently, the number and diameter of the aggregates, and the number of viable and nonviable cells were assessed three times for each RCCS-D. The concentrations were adjusted to have cell aggregates equivalent to 0.25 × 10⁶ or 0.5 × 10⁶ viable cells per 0.5 ml suspension for intra-portal injection in group C or D, respectively. The second volume was trypsinized and washed in PBS twice, to completely desaggregate the cells for accurate counting. At the end of this procedure, all cells were found to be de-aggregated but nonviable. Therefore, the total number of (non-viable) cells counted after trypsinization was reduced with the number of nonviable cells counted before trypsinization (number of “viable” cells after trypsinization). This subtotal number of cells should be equal to the sum of the aggregated and non-aggregated viable cells in the RCCS-Ds before trypsinization. If the estimated amount of cells in the RCCS-Ds is correct, the ratio of both numbers should be 1.

Experimental Setup

Cancer cell suspension was injected as a volume of 0.5 ml into the portal vein through a catheter (Terumo needle 26G × ½), and flushed with 0.5 ml of physiological saline solution. The site of injection was covered with resorbable hemostatic sponge.

A pilot study was conducted to define the lowest number of single non-aggregated viable cancer cells that cause macroscopic liver metastases at 8 weeks after intra-portal injection. Twenty-four rats were divided into 6 equal groups. Groups were injected with an increasing number of DHD/K12/TRb cancer cells i.e., 10⁵, 0.25 × 10⁶, 0.5 × 10⁶, 10⁶, 2 × 10⁶, and 5 × 10⁶. In this pilot study macroscopic liver metastases were found in 7/12 and in 2/12 rats after intraportal injection of >0.5 × 10⁶ and ≤0.5 × 10⁶ cancer cells, respectively. Based upon these findings, the lowest number of cancer cells causing macroscopic liver metastases was defined as the number of cancer cells varying between 0.25–0.5 × 10⁶.

In the following experiments 0.25 or 0.5 × 10⁶ DHD/K12/TRb cancer cells were injected via the portal vein, either as viable single nonaggregated cells or as cell aggregates:

Group A: 30 rats injected with 0.25 × 10⁶ viable single cancer cells. Group B: 20 rats injected with 0.5 × 10⁶ viable single cancer cells. Group C: 20 rats injected with cell aggregates equivalent to 0.25 × 10⁶ viable cells. Group D: 19 rats injected with cell aggregates equivalent to 0.5 × 10⁶ viable cells.

The effect of immunosuppression with cyclosporin A for 4 weeks (subcutaneous injection; 10 mg/kg/day) on the transformation from micro- into macrometastases was assessed macroscopically (and histologically) in rats that did not develop macroscopic metastases at 8 weeks after cancer cell injection.

Histopathology

Five rats were injected with 0.5 × 10⁶ viable non-aggregated cancer cells, and another five with aggregated cells. In these 10 rats a total heptectomy was performed within 15 min after cancer cell injection via the portal vein. The entire liver was sliced at 2-mm intervals, embedded in paraffin, and stained with hematoxylin–eosin. For each rat, 10 liver sections were analyzed at random. An independent pathologist (TR) examined the liver sections to localize the injected cancer cells, without knowing to which group the specimens belonged. Both number and location of aggregated and non-aggregated cancer cells were assessed under light microscopy.

Statistical Analysis

Analysis of the statistical significance of differences between groups of data were performed using two-tailed Fisher exact test. A
the different groups (Table 3). However, this difference was not statistically significant ($P = 0.19$). As metastatic efficiency in group D reached a high level (81%), no randomization for cyclosporin A treatment was performed in this group.

**Histopathology**

In all rats injected with $0.5 \times 10^6$ viable aggregated cancer cells, clusters of viable cancer cells were located in portal vein branches, containing a mean ± SD number of $67 \pm 49$ cancer cells. In only 1 out of 5 rats injected with $0.5 \times 10^6$ viable non-aggregated cancer cells, a single group of 20 picnotic cancer cells were found in a portal branch. Comparable numbers of grouped picnotic cancer cells were found to be located in centrolobular (8 $\pm$ 7 versus 10 $\pm$ 8) and midzonal sinusoids (13 $\pm$ 12 versus 10 $\pm$ 8) in rats injected with non-aggregated cancer cells, as well as in rats injected with aggregated cells.

### RESULTS

#### Cell Count

The mean ± SD diameter of aggregates was $57 \pm 24 \mu m$, with a minimum of $20 \mu m$ and a maximum of $200 \mu m$. Viable cancer cells in RCCS-Ds consisted in $13 \pm 4\%$ single or nonaggregated and $87 \pm 4\%$ aggregated cells. Mean viability of cells in RCCS-Ds was $77.9 \pm 2.1\%$, whereas no viable cells could be counted after the trypsinization process as mentioned in the methods section. The mean ratio of the estimated number of viable cells before trypsinization/the number of “viable” cells after trypsinization in the RCCS-Ds was found to be 1.13 (95% CI 0.8–1.47; Table 1).

#### Metastatic Efficiency

Nine rats were not eligible because of mortality because of ether-anesthesia or to intraoperative bleeding at the site of injection (four in group A; one in B; one in C; and three in D).

Liver metastases were found most frequently after intraportal injection of cell aggregates equivalent to $0.5 \times 10^6$ viable cells (group D; Table 2). Two-tailed Fisher Exact test for $2 \times 4$ table revealed a significant difference in the development of liver metastases between the different groups ($P = 0.0002$), with the most significant difference between group D and B ($P = 0.002$). No difference was found between groups A, B, and C. The development of peritoneal metastases was low and comparable in all groups ($P = 0.11$).

Treatment with cyclosporin A resulted in liver metastasis formation in 5 of 24 (20.8%) rats compared to 1 of 21 (4.7%) in the control group (Table 3). However, this difference was not statistically significant ($P = 0.11$). As metastatic efficiency in group D reached a high level (81%), no randomization for cyclosporin A treatment was performed in this group.

#### DISCUSSION

Intraportal injection of cells derived from a colon carcinoma cell line is the experimental rat model most often used in studying colorectal liver metastases. This model produces up to 100% hepatic metastases 6 weeks after intra-portal injection of $10–20 \times 10^6$ cancer cells [14].

In the present study an 81% metastatic efficiency in the liver was observed after intra-portal injection of cell aggregates equivalent to $0.5 \times 10^6$ cancer cells. A significantly lower metastatic efficiency was found after the injection of $0.5 \times 10^6$ non-aggregated, and $0.25 \times 10^6$ aggregated or nonaggregated cancer cells. Thus, aggregated cancer cells appear to have a higher metastatic efficiency, although a critical number of cancer cells are necessary i.e., $0.5 \times 10^6$. It has to be taken into account that the number of injected aggregated cancer cells cannot be counted exactly because any manipulation of the aggregated cells before the injection will jeopardize both the number and viability of the cells. Therefore, an estimation of the number of aggregated cells was performed. The ratio of the estimated number of viable cells before trypsinization/the number of “viable” cells after trypsinization in the RCCS-Ds was found to be 1.13 (95% CI 0.8–1.47; Table 1). This can be explained by the fact that nonviable cells within aggregates were not taken into account. Indeed, light microscopic assessment of the viability of aggregated cells could not be performed with appropriate confidence, so that an overestimation of the cell viability in the RCCS-Ds can be expected.

In their work Panis et al. used syngeneic male BD IX rats injected intra-portal with DHD/K12 colorectal cancer cell aggregates obtained from the incubation of $0.5 \times 10^6$ cancer cells to develop macro-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Estimation of the Number of Viable Aggregated and Single or Nonaggregated Cancer Cells in RCCS-Ds</th>
<th>Mean</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregates/ml</td>
<td>47222</td>
<td>31149–63294</td>
</tr>
<tr>
<td>Mean diameter of aggregates/μm</td>
<td>57</td>
<td>31–82</td>
</tr>
<tr>
<td>Cells/aggregate</td>
<td>38</td>
<td>25–51</td>
</tr>
<tr>
<td>Viable aggregated cells/ml/RCCS-D</td>
<td>1704475</td>
<td>1195138–2213812</td>
</tr>
<tr>
<td>Viable single cells/ml/RCCS-D</td>
<td>291667</td>
<td>250000–333333</td>
</tr>
<tr>
<td>Non-viable single cells/ml/RCCS-D</td>
<td>633333</td>
<td>616667–650000</td>
</tr>
<tr>
<td>Total viable cells/ml/RCCS-D</td>
<td>1938799</td>
<td>1362903–2514695</td>
</tr>
<tr>
<td>Total cells/ml after trypsinization</td>
<td>2259722</td>
<td>1719254–2800191</td>
</tr>
<tr>
<td>“Viable” cells/ml after trypsinization</td>
<td>1760324</td>
<td>1339299–2181348</td>
</tr>
<tr>
<td>Ratio of viable cells in RCCS-D/“viable” cells after trypsinization</td>
<td>1.13</td>
<td>0.80–1.47</td>
</tr>
</tbody>
</table>

$P$ value of ≤0.05 was considered statistically significant. All analyses were performed with the statistical package StatSoft Statistica (version 6).
scopic liver metastases from dormant cancer cells after immunosuppression [3]. This means that after an incubation period of 2 days, the potential number of cells injected may be around $2 \times 10^6$, because doubling time for DHD/K12/TRb cells is 25.9 hr [15]. In the present study, cyclosporin A treatment did not result in a significantly increased metastasis formation of assumed “dormant” cancer cells in the liver. This may be related to the limited number of animals studied. Also, the final number of cancer cells injected was not higher than $0.5 \times 10^6$. Intraportal injection of $0.25 \times 10^6$ aggregated or nonaggregated, and $0.5 \times 10^6$ non-aggregated DHD/K12/TRb cancer cells may therefore not be sufficient enough to enable cancer cells to become dormant in the liver, since the majority of cells injected through the portal vein are cleared in the hepatic microvasculature.

The development of metastasis is a highly selective process favoring the survival of a minor subpopulation of metastatic cells. Cancer cells from this subpopulation have to complete a sequence of interrelated steps to produce clinically relevant distant metastases. Failure to complete one or more steps disrupts the linkage in this metastatic cascade and eliminates the cell. An essential step in the formation of liver metastasis is the adherence of tumor cells to the microvasculature of the liver. The trapping of cells in the hepatic circulation does not ensure their survival. Cell surface adhesion molecules specific for receptor molecules on the hepatic endothelial cells and extracellular matrix are necessary for attachment and subsequent growth in the liver. DHD/K12 colon cancer cells express on their cell surface a glycoprotein called pE4 that is a member of the immunoglobulin supergene family functioning as an intercellular adhesion molecule [16]. The finding that aggregated DHD/K12/TRb colon cancer cells have a higher metastatic efficiency compared to non-aggregated cells can therefore be explained by the combination of these phenomena. Aggregated cancer cells may remain in large clusters of viable cells, and trapped in the portal branches of the liver where they get attached to the hepatic endothelial cells. Here they may be able to evade host defense mechanisms since these are concentrated in the sinusoids. Nonaggregated cancer cells, however, may be unable to form clusters of viable cells and pass through the portal branches into the centrolobular and midzonal sinusoids of the liver, where they were found to be picnotic.

In the present study up to $0.5 \times 10^6$ aggregated colon cancer cells are shown to be necessary to have a high metastatic efficiency in the liver. Turnbull propagated the “no-touch isolation technique” for colon cancer surgery to avoid intra-operative hematogenous cancer cell dissemination [17]. Much fewer cancer cells were found in portal blood during the no-touch technique than during conventional procedures. However, in a controlled prospective trial no improvement in survival could be shown in favor of the no-touch technique. Only a tendency for reduction in the number of, and time to, occurrences of distant metastases was found.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Liver Metastases</th>
<th>Peritoneal Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: 0.25 $\times 10^6$ viable single cancer cells</td>
<td>26</td>
<td>7 (27%)</td>
<td>1 (4%)</td>
</tr>
<tr>
<td>B: 0.5 $\times 10^6$ viable single cancer cells</td>
<td>19</td>
<td>3 (16%)</td>
<td>3 (16%)</td>
</tr>
<tr>
<td>C: cell aggregates equivalent to 0.25 $\times 10^6$ viable cells</td>
<td>19</td>
<td>6 (32%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>D: cell aggregates equivalent to 0.5 $\times 10^6$ viable cells</td>
<td>16</td>
<td>13 (81%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Liver Metastases</th>
<th>Peritoneal Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: 0.25 $\times 10^6$ viable single cancer cells</td>
<td>19</td>
<td>1/9 (11.1%)</td>
<td>0/9 (0%)</td>
</tr>
<tr>
<td>B: 0.5 $\times 10^6$ viable single cancer cells</td>
<td>13</td>
<td>0/9 (0%)</td>
<td>2/6 (33.3%)</td>
</tr>
<tr>
<td>C: cell aggregates equivalent to 0.25 $\times 10^6$ viable cells</td>
<td>13</td>
<td>0/6 (0%)</td>
<td>1/7 (14.3%)</td>
</tr>
<tr>
<td>Total A + B + C</td>
<td>45</td>
<td>1/21 (4.7%)</td>
<td>12/24 (50.0%)</td>
</tr>
</tbody>
</table>
Liver metastases was seen in the no-touch isolation group [18]. Quantification of cancer cell dissemination was not performed in these studies. Further study in quantification of cancer cell dissemination before and during curative resection of primary colorectal cancer is needed, to better select and treat patients at high risk for the development of recurrent and/or metastatic disease. In conclusion, aggre-

FIG. 1. Light microscopic view of nonaggregated DHD/K12/TRb colon cancer cells (a) before intraportal injection and (b) after intraportal injection in the liver of BD IX rats (hematoxylin–eosin staining). A small group of tumor cells with a picnotic nucleus is present near the central vein branch (arrow).
gated colon cancer cells have a higher metastatic efficiency in the liver compared with nonaggregated cells, although a critical number of cancer cells are necessary.

ACKNOWLEDGMENTS

We thank G. Basha and T. Crabbé for their assistance in cancer cell culturing.
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