Phosphoinositide 3-Kinase Accelerates Postoperative Tumor Growth by Inhibiting Apoptosis and Enhancing Resistance to 
Chemotherapy-induced Apoptosis

NOVEL ROLE FOR AN OLD ENEMY*§

Received for publication, December 30, 2004, and in revised form, February 15, 2005
Published, JBC Papers in Press, March 1, 2005, DOI 10.1074/jbc.M414696200

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Tumor removal remains the principal treatment modality in the management of solid tumors. The process of tumor removal may potentiate the resurgent growth of residual neoplastic tissue. Herein, we describe a novel murine model in which flank tumor cytoreduction is followed by accelerated local tumor recurrence. This model held for primary and recurrent tumors generated using a panel of human and murine (LS174T, DU145, SW480, SW640, and 3LL) cell lines and replicated accelerated tumor growth following excisional surgery. In investigating this further, epithelial cells were purified from LS174T primary and corresponding recurrent tumors for comparison. Baseline as well as tumor necrosis factor apoptosis-inducing ligand (TRAIL)-induced apoptosis were significantly reduced in recurrent tumor epithelia. Primary and recurrent tumor gene expression profiles were then compared. This identified an increase and reduction in the expression of p110γ and p85α class Iα phosphoinositide 3-kinase (PI3K) subunits in recurrent tumor epithelia. These changes were further confirmed at the protein level. The targeting of PI3K ex vivo, using LY294002, restored sensitivity to TRAIL in recurrent tumor epithelia. In vivo, adjuvant LY294002 prolonged survival and significantly attenuated recurrent tumor growth by greatly enhancing apoptosis levels. Hence, PI3K plays a role in generating the antiapoptotic and chemoresistant phenotype associated with accelerated local tumor recurrence.

For over a century, tumor removal has remained the principal treatment modality in the management of solid tumors (1, 2). Increasing evidence indicates that tumor removal exerts a potentiating effect on residual neoplastic disease (1–7).

Following cancer surgery, patients frequently retain neoplasia in a micro- or macroscopic form. This fact, coupled with the possibility that the process of tumor removal may potentiate tumor growth, has major implications for patients with residual disease. Following rectal cancer excision, the overall incidence of local recurrence is 30% (with the range varying from 10 to 50%) (8). 75% of patients who die following excisional surgery for rectal cancer, do so from local recurrence (8). At presentation, 25% of patients with colorectal cancer have synchronous hepatic metastases. Despite complete excisional surgery, a further 25% develop metastatic disease. Greater then 50% of patients undergoing colorectal tumor excision, harbor or shed circulating tumor cells either prior to or during the procedure (9, 10). These facts indicate that residual neoplasia occurs in a variety of forms and in a significant proportion of patients, following surgery for rectal neoplasia. The concept that tumor removal could potentiate recurrent tumor growth thus increases significantly in relevance.

Extensive clinical and experimental evidence indicates that tumor removal adversely alters the growth kinetics of residual neoplasia (11–18). Tumor cell proliferation is increased in local tumor recurrences following curative-intent excisional surgery (3, 7). This finding indicates that surgical treatment undertaken in a cancer patient could promote recurrent tumor growth (3, 7). Chang et al. (19) demonstrated a strong overlap between genes involved in tumor progression and those involved in wound healing. Tumor progression is linked with extracellular mediators that are increased during wound healing (i.e. tumor necrosis factor-α, interleukin-6, and transforming growth factor-β) (20). Inflammation is a well known risk factor for neoplasia exerting a promoter-like effect on tumors already present (21). These effects are mediated through extracellular factors such as platelet-derived growth factor, vascular epidermal growth factor, and insulin-like growth factor and increased activities in nuclear factor kappa B and phosphoinositide 3-kinase pathways, among others (21, 22). Wound formation, inflammation, and healing are unavoidable sequelae of the surgical process. This overlap underscores a relationship between tumor removal and recurrent tumor growth.

To date, most studies that have evaluated accelerated postoperative tumor growth focused on extracellular mediators (i.e. endostatin, angiostatin, platelet-derived growth factor, and vascular epidermal growth factor) and end points such as proliferation and apoptosis. Interestingly, changes in apoptosis have been repeatedly noted during the postoperative period (4, 5, 23–26). Shortly following primary tumor removal, diminished extracellular endostatin and angiostatin result in reductions in apoptosis and accelerations in metastasis formation (1, 5, 24). Tumor dormancy is a function of the balance between apoptosis and proliferation (6). This balance is disrupted after tumor removal, i.e. when endostatin levels are reduced and
angiogenesis promoted (6). Postoperatively, increases in serum endotoxin are directly associated with increased vascular epidermal growth factor levels (24, 25). In vitro, vascular epidermal growth factor reduces apoptosis by increasing BCL2 levels (24, 25). Also postoperatively, increases in wound-related growth factors result in reduced apoptosis and simultaneously drive increased cell proliferation. To date however, no studies have addressed the intracellular events that must underpin these endpoints.

The aim of this study was to characterize recurrent tumor growth during the postoperative interval. Locally recurrent tumors developed in an accelerated manner. As this was associated with reduced levels of apoptosis, this reproduced accelerated postoperative tumor growth and provided a novel template to further characterize the intracellular basis for this phenomenon. Epithelia purified from local tumor recurrences demonstrated enhanced resistance to TRAIL1-induced apoptosis. Several transcriptional differences separated primary and recurrent tumor epithelia. These included collective changes in the expression of genes encoding for the class Ia phosphoinositide 3-kinase complex. The targeting of the phosphoinositide 3-kinase significantly slowed recurrent tumor growth, restored sensitivity to TRAIL, improved long term survival, and restored an apoptotic phenotype similar to that seen in primary tumors.

MATERIALS AND METHODS

Cell Lines, Animals, and Reagents—Cell culture reagents were purchased from BioWhittaker Europe. Flask tumors were generated using the following panel of cell lines: LS174T, SW480, and SW620 (human colorectal adenocarcinoma), DU145 (human prostatic adenocarcinoma), and 3LL (murine bronchial adenocarcinoma), respectively (all from the American Type Culture Collection). Cells were cultured to 80% confluence in the appropriate medium (e.g. in Eagle’s minimum essential medium for LS174T cells, RPMI 1640 for DU145 cells) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin sulfate, and 2 mM l-glutamine, in a humidified atmosphere of 5% CO2 in air at 37 °C. At 80% confluency cell cultures were trypsinized using 0.25% trypsin-EDTA and washed three times in phosphate-buffered saline. Cells were counted and resuspended in phosphate-buffered saline at a concentration of 1 × 106/ml for inoculation and primary tumor generation.

Weight-matched male mice (Harlen, UK) were used (age, 6–8 weeks; weight, 26 ± 2 g) in all studies. Animals were housed in a licensed biomedical facility in the University College of Cork, and following acclimation animals were maintained in an air-conditioned room at 21 °C with 50% relative humidity using a 12-h light-dark cycle regimen. All animal-based experiments were performed by an authorized licensed according to guidelines as set out by the Ministry for Health in Ireland.

Primary Tumor Cytoreduction Model—Flank tumors were first generated following inoculation with LS174T cells in mnu1nu1 nude mice (n = 40). All animals received a flank tumor injection of 200 μl of cells (1.0 × 106 cells/mouse). Tumor volumes were measured using longest and shortest perpendicular diameters (designated a and b, respectively). Tumor volume was calculated using the formula volume = π/6 × a × b2 (18). After 3 weeks, animals were anesthetized using intraperitoneal xenlapar® and narcat® (Chassot AG). Next, under sterile conditions, primary tumors underwent near-maximal cytoreduction. In each case, a tumor piece measuring ~1 (1 × 1 × 1 mm)3 remained in situ. The aim of leaving a small amount of neoplastic tissue was firstly to ensure that a recurrent tumor developed and secondly to mimic near maximal tumor cytoreduction in the clinical setting. Hence, this model pertains to the clinical scenario wherein all attempts to remove a tumor are made but where small macroscopic scopic feed of residual tissue are inadvertently retained. Animals were recovered as before, and recurrent tumor volumes measured. After 2 weeks all recurrent tumors were completely excised (n = 40) and animals euthanized via cervical dislocation. Identical experiments were performed using the SW480 and SW620 human colorectal adenocarcinoma cell lines in mfl nu1nu1 nude mice (n = 10 for both cell lines).

In the second experiment, DU145 flank tumors were generated in SCID mice (n = 8) following subcutaneous inoculation with 1.0 × 106 cells in 200 μl (1.0 × 106 cells/mouse). Primary tumor volumes were determined as described above. DU145 flank tumors were slow growing (requiring over 5 weeks to reach a volume of 1.5 cm3). Following several weeks, complete excision was performed after which there was no macroscopic evidence of residual tumor in situ. The volumes of those tumors that recurred were recorded. Flank tumors were also generated using the 3LL cell line, in C57Bl/6 mice (1.0 × 106 cells/mouse) (n = 10 mice). 3LL tumor volumes were measured as described above. Where recurrent tumors developed, following complete excisional surgery, their volume was again determined. In the case of each of these models no macroscopic evidence of neoplastic disease was evident at the time of primary tumor excision. These models thus approximated to the clinical scenario wherein following complete excisional surgery, residual neoplastic material was then inoculated into a mouse.

Having obtained ethical approval and informed consent an human explant study was next performed. To determine the growth characteristics of a human tumor both pre- and post-cytoreduction, tumor mor-
cells (n = 6) were obtained from a human colorectal adenocarcinoma and transplanted into the flank of nude mice (n = 6). Following 3 weeks of growth, near maximal cytoreduction was performed and tumor regrowth was compared with primary tumor growth.

Apoptosis in Primary and Recurrent Tumors—The aim of the present study was to characterize recurrent tumor growth when neoplasia is retained in either macroscopic or microscopic form. For molecular studies involving the harvest of RNA, it was imperative that these be performed on the first experimental model, i.e. when macroscopic neo-
plasia was deliberately retained. The reason for this is that, using this model, primary and recurrent tumors are guaranteed to develop in a predictable fashion. This facilitates comparative studies between primary and recur-
current tumors. Hence all the remaining experiments pertained to the first animal model, i.e. the LS174T/mflnu1nu1 cytoduction model.

Tumor specimens were fixed overnight in formalin before being wax-embedded and sectioned at 8 μm. Sections from primary and recurrent tumors were stained with hematoxylin and eosin (H&E). Morphological features were determined and compared by pathologists who were blinded to the nature of the tissue specimen. Apoptotic cells were identified based on morphological changes, and an apoptotic index was established for both primary and recurrent tumors. Apoptotic cells were further identified using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin end labeling) immunohistochemistry, as previously described (see Supplementary Materials) (27). A cell with both TUNEL immunoreactivity and morphological features of apoptosis was designated as apoptotic. Apoptotic indices were calculated as the mean number of identified cells per ten high power fields. Apoptotic indices were then compared between primary and corresponding recur-
current tumors.

To further characterize differences in levels of apoptosis between primary and recurrent tumors, epithelia from both primary and corresponding recur-
current tumors. To detect apoptosis cells were centrifuged at 1500 rpm for 7 min at 4 °C and then resuspended in 0.5 ml of fluorochrome solution (50 μg/liter propidium iodide, 3.4 μm/liter sodium citrate, 1 μm/liter Tris, 0.1 μm/liter EDTA, and 0.1% Triton x100). Following 6-h incubation in darkness at 4 °C, apoptosis was measured by a FACSscan flow cytometer (BD Biosciences, Mountain View, CA). Forward and side scatter was measured, and the fluorescence of FL-2 was registered on a linear scale. A minimum of 50,000 events were collected and analyzed on CellQuest software (BD Biosciences).

Tumorigenicity and Metastatic Potential in Primary and Recurrent Tumor LS174T Epithelial Isolates—LS174T primary and recurrent tumors were generated as described above. Following tumor harvest, a single cell suspension was generated as described in Supplementary Materials. The tumorigenicity of epithelia isolated from primary and recurrent tumors was then tested in vivo. Eight-week-old mice were inoculated subcutaneously with the flank of nude mice (1.0 × 106 cells/animal). The volumes of tumors generated from primary and recurrent tumor epithelial isolates were determined and compared. All animals were sacrificed following 21 days tumor growth after which apoptotic indices

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1 The abbreviations used are: TRAIL, tumor necrosis factor apoptosis-inducing ligand; RT, recurrent tumor; H&E, hematoxylin and eosin; MSD, mean signal density or fold difference; PI3K, phosphatidylinositol 3-kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.
were again established using TUNEL immunohistochemistry. To compare the metastasis-forming potential of primary and recurrent tumor epithelial isolates, these were re-inoculated into the spleens of nude mice (1.0 × 10^6 cells/animal; n = 3), using a technique previously described (28). Mice were sacrificed after 6 weeks, following which metastatic burden was then assessed.

**Gene Microarrays and Bioinformatic Evaluation**—Total RNA was harvested, from purified primary and recurrent tumor epithelial isolates. RNA was generated using TRIzol (GiboBrl), as previously described, with slight modifications (a second TRIzol precipitation step was incorporated) (29). RNA was evaluated using a combination of spectrophotometry and RNA gel fragmentation. Gene microarray hybridization was performed according to the protocol outlined in the Human Lifegrid™ 1.1 Product Manual (IncyteGenomics, 2000) with slight modifications. Nylon filter microarrays were used consisting of 8400 human PCR products. In brief, 32P-labeled probes were prepared using total RNA harvested from purified primary and recurrent tumor parenchyma (n = 3 arrays for each). Ambion Strip-EZ reagents were used in the preparation of labeled probes. These were then hybridized for 5 days before visualization on a PhosphorImager, image capture using Imagene© and evaluation using Arrayvision software©. Finally, each array was stripped of labeled probe and reconstituted as described previously.

Microarrays were generated from epithelial cells purified from three primary tumors and their corresponding three recurrent tumors. This generated six sets of expression data, three from primary tumor epithelia and three from recurrent tumor epithelia. The data from each were processed and evaluated as described in the Supplementary Materials. This generated a numerical ratio (the mean signal density or fold difference, i.e. MSDr) that indicated the extent to which each gene differed in expression between primary and recurrent tumor epithelial isolates. For the purposes of presentation a MSDr of 2 or 0.5 was chosen as the cut-off for significant expression differences. All MSDr values were statistically compared with determine whether differences detected were statistically significant. Statistical analysis was performed using Sigmastat© (Jandel Scientific, San Rafael, CA). Differences between means were determined using the t test, if groups passed a normality or equal variance test. Otherwise the Mann-Whitney Rank Sum test was used with, p < 0.05 taken as significant.

**Western Blot Analysis**—As changes outlined in the array studies were confirmed statistically, we confined confirmatory protein-based studies to a select number of proteins. These included the p85 and p110 regulatory and catalytic subunits of phosphoinositide 3-kinase, respectively, as well as phosphorylated Akt. Western blots were performed as
corresponding primary tumors both at and prior to excision (†, p < 0.001; ‡, p = 0.026; ∥, p < 0.001; and Y, p = 0.029, Fig. 1A). To determine if these observations were LS174T cell-specific, the experiments were repeated using the SW480 and SW620 cell lines. Similar differences in both the rate and pattern at which primary and recurrent tumors increased in volume were observed (data not shown). To determine if this phenomenon was exclusive to colorectal tumors, the experiment was repeated using the DU145 human prostate adenocarcinoma cell line. Following complete DU145 xenograft excision recurrent tumors developed in four of eight animals. This model was representative of complete tumor excision and depicted recurrent tumor growth when microscopic foci of residual neoplasia are retained following excisional surgery. DU145 primary tumor xenografts were slow growing, requiring 46 days to reach volumes >1.5 cm³ (Fig. 1B). Recurrent tumors again grew more rapidly, reaching over 1.5 cm³ within 10 days of excision (†, p = 0.02; ‡, p < 0.001; and Y, p = 0.3, Fig. 1B).

The above models involved immuno-compromised (nude and SCID) mice. To determine if the same effect were observed in non-immuno-compromised mice the study was repeated excising 3LL flank tumors generated in C57BL/6J mice. Following complete excision, recurrent tumors were again seen to develop at an accelerated rate (data not shown). To determine if human tumors exhibited a similar phenomenon tumor morcell (n = 6) were harvested from a human colorectal adenocarcinoma (Dukes A). These were explanted subcutaneously into mflnu nu mice (n = 6). Three of the six implants generated primary tumors. These were slow growing and developed along an exponential growth curve (Fig. 1C). The remainder regressed, being necrotic when examined histologically. Following cytoreduction, recurrent tumors developed rapidly (Fig. 1C). Early during their growth, the volume of recurrent tumors exceeded primary tumor volumes both at and prior to excision (†, p < 0.001; ‡, p < 0.001; Y, p = 0.029; and Y, p = 0.4, Fig. 1C). Hence, the growth kinetics of primary and corresponding recurrent tumors differ, and this is seen irrespective of cell type and immune
status. Moreover, this phenomenon is repeated for explanted human tumor specimens.

**Apoptosis Is Reduced in Recurrent Tumors**—Apoptotic indices were separately established using H&E, TUNEL immunohistochemistry, and propidium iodide fluorescence for both primary and recurrent tumors. The H&E (†, \( p < 0.001 \); t test) and TUNEL (‡, \( p = 0.002 \); Fig. 2) apoptotic indices were significantly reduced in recurrent tumors. To assess this difference further, post-sorting apoptosis among viable LS174T cells that were harvested from primary and recurrent tumors, was compared. Uptake of propidium iodide was seen to be significantly reduced within recurrent tumor epi-

![Fig. 4. Bar chart summarizing levels of apoptosis in primary and corresponding recurrent tumor epithelial isolates following incubation with recombinant human TRAIL at 100, 500, and 1000 ng/ml concentrations (all \( p < 0.001 \); t test). c = control in vitro LS174T cells; P and R refer to primary and recurrent tumor epithelia, respectively.](image)

**Table I**

*Select genes induced in recurrent tumors as outlined using oligonucleotide microarrays*

The MSD, (i.e. -fold difference) represents the ratio of the primary tumor mean signal density, for each gene, to the recurrent tumor mean signal density. A ratio of <0.5 represents gene induction within recurrent tumor epithelia. Specific \( p \) values are also included. GenBank™ and Incyte ID numbers are indicated, and the genes are listed according to the level to which they are induced within recurrent tumor epithelia (i.e. those induced the strongest are at the top of the list).

| Incyte gene ID No. | GenBank™ gene ID No. | Gene product | MSD ratio | \( p \) Value |
|-------------------|----------------------|--------------|-----------|-------------|
| 1569138           | Hs.168212            | Kinesin family member 3B | 0.1630728 | 0.001       |
| 887867            | Hs.78146             | Platelet/endothelial cell adhesion molecule (CD31 antigen) | 0.334378 | <0.001       |
| 672836            | Hs.117977            | Kinesin 2 (60–70 kDa) | 0.104415 | <0.001       |
| 2559673           | Hs.227817            | BCL2-related protein A1 | 0.075606 | 0.009       |
| 2368776           | Hs.24439             | Ring finger protein (C9H4 type) 8 | 0.239846 | 0.005       |
| 3495906           | Hs.79019             | Baculoviral IAP repeat-containing 1 | 0.217754 | 0.001       |
| 3536740           | Hs.167218            | BarH-like homeobox 2 | 0.202155 | <0.001       |
| 566886            | Hs.695               | Cystatin B (stefin B) | 0.192639 | <0.001       |
| 1976602           | Hs.160011            | Catenin (cadherin-associated protein), delta 1 | 0.187816 | 0.012       |
| 1747050           | Hs.19717             | Interleukin 10 | 0.330733 | 0.005       |
| 2503995           | Hs.183418            | Cell division cycle 2-like 1 (PITSLRE proteins) | 0.317615 | 0.01       |
| 1994460           | Hs.211579            | Melanoma adhesion molecule | 0.298578 | 0.005       |
| 3053958           | Hs.2134              | TNF receptor-associated factor 1 | 0.295040 | <0.001       |
| 3176179           | Hs.246381            | CD68 antigen | 0.283813 | 0.01       |
| 61099             | Hs.51                | Phosphatidylinositol glycan, class A (paroxysmal nocturnal hemoglobinuria) | 0.279423 | 0.01       |
| 2200368           | Hs.171271            | Catenin (cadherin-associated protein), \( \beta 1 \) (88 kDa) | 0.265423 | <0.001       |
| 1643474           | Hs.219140            | Natriuretic peptide precursor B | 0.380261 | 0.005       |
| 1905732           | Hs.211299            | Thioredoxin, mitochondrial | 0.377665 | 0.045       |
| 2174773           | Hs.125380            | Bladder cancer overexpressed protein | 0.368021 | 0.001       |
| 1864543           | Hs.162008            | Phosphoinositide 3-kinase, catalytic, delta polypeptide (PI3KCD) | 0.365122 | 0.045       |
| 1735623           | Hs.183236            | CDC10 (cell division cycle 10, Saccharomyces cerevisiae, homolog) | 0.359691 | 0.01       |
| 3999519           | Hs.90957             | TNF receptor-associated factor 6 | 0.451703 | 0.02       |
| 160822            | Hs.11899             | 3-Hydroxy-3-methylglutaryl-Coenzyme A reductase | 0.451089 | 0.001       |
| 4813875           | Hs.79006             | Deoxymyristidylate kinase (thymidylate kinase) | 0.448215 | 0.001       |
| 2884153           | Hs.75746             | Aldyde dehydrogenase 6 | 0.442456 | 0.02       |
| 2399995           | Hs.15767             | Citron ( rho-interacting, serine/threonine kinase 21) | 0.442487 | 0.001       |
| 1427623           | Hs.195799            | Microsomal triglyceride transfer protein (large polypeptide, 88 kDa) | 0.441575 | 0.005       |
| 2930175           | Hs.33565             | Expressed sequence tags | 0.441522 | 0.005       |
| 1929073           | Hs.119523            | Transform-2a (hrtr-a2a) | 0.440194 | 0.05       |
| 1994609           | Hs.9676              | Uncharacterized hypothalamus protein HT012 | 0.440153 | 0.01       |
| 1722903           | Hs.112078            | KIAA0339 gene product | 0.439397 | 0.01       |
| 1701814           | Hs.36993             | Gastrulation brain homeo box 1 | 0.439218 | 0.04       |
| 1989922           | Hs.155120            | rho/rac guanine nucleotide exchange factor (GEF) 2 | 0.434363 | 0.05       |
| 1623214           | Hs.122764            | BRCA1-associated protein | 0.424154 | 0.01       |
| 3152987           | Hs.79057             | Heat shock 60-kDa protein 1 (chaperonin) | 0.415409 | 0.01       |
| 3681722           | Hs.75415             | \( \beta 2 \)-Microglobulin | 0.409849 | 0.01       |
| 3000548           | Hs.104481            | Nck, Ash, and phospholipase C-binding protein | 0.4052 | 0.05       |
thelial isolates (γen), \( p = 0.001 \), Fig. 2). These findings further support the observation that apoptosis was reduced in recurrent tumors.

**Ex Vivo Assays Confirm Enhanced Spontaneous Primary and Metastatic Tumorigenicity in Recurrent Tumor Epithelia—**

The tumorigenicity of primary and recurrent tumor human epithelial artigenic-positive cells was compared. Primary and recurrent tumor epithelial cells were isolated to purity and non-viable cells washed clear. Equal numbers of viable cells were inoculated in all animals. Tumor nodules were visible in recurrent tumor epithelial cells were isolated to purity and these were re-inoculated via intrasplenic injection in a protocol containing between 8 \( \times 10^5 \) and 1 \( \times 10^6 \) recurrent tumor cells (columns RT(D) and RT(E), Fig. 3B).

**Chemosensitivity to TRAIL Is Enhanced in Recurrent Tumor Epithelial Isolates—**

Tumor necrosis factor apoptosis-inducing ligand (TRAIL) is a potent inducer of apoptosis in neoplastic cells. The sensitivity of primary and corresponding recurrent tumor epithelial isolates to TRAIL was determined (Fig. 4). Primary tumor cells were sensitive to TRAIL. TRAIL led to a dose-dependent increase in apoptosis (Fig. 4). When cells isolated from recurrent tumors were incubated with TRAIL at 100, 500, and 1000 ng/ml, apoptosis levels were significantly reduced relative to primary tumor counterparts (all \( p < 0.001 \), Fig. 4). When TRAIL was used at 100 ng/ml, no increase in apoptosis was observed over controls (Fig. 4). At higher doses an increase in apoptosis was seen, but this was significantly reduced relative to that seen for primary tumor isolates treated in the same manner (Fig. 4).

**Differential Gene Expression in Epithelia Purified from Primary and Corresponding Recurrent Tumors—**

Gene expression profiles were generated from purified primary and recurrent tumor epithelia for comparison. A total of 162 genes was significantly upregulated within recurrent tumors (i.e. the MSD, was >5). A selection of those genes that were up-regulated is given in Table I. Conversely, 272 genes were down-regulated within current tumor epithelial isolates (i.e. the MSD, was <2). A selection of genes down-regulated is given in Table II. A clear trend emerged when the expression of genes encoding subunits of class Ia phosphoinositide 3-kinase (PI3K), were compared

### TABLE II

| Invitrogen gene ID No. | GenBank Gene ID No. | Gene product | MSD ratio | \( p \) Value |
|------------------------|---------------------|--------------|-----------|--------------|
| 1277992                | Hs.57732            | Mitogen-activated protein kinase 11 | 16.293198 | 0.001        |
| 2044961                | Hs.457              | Fucosyltransferase 7 (α-L-fucosyltransferase) | 14.315922 | 0.005        |
| 2372464                | Hs.99423            | ATP-dependent RNA helicase | 13.061117 | 0.005        |
| 142949                 | Hs.120996           | Serine/threonine kinase 17B (apoptosis-inducing) | 11.076228 | 0.01         |
| 3472502                | Hs.1200             | Arachidonate 12-lipoxygenase | 10.48929 | 0.001        |
| 1879041                | Hs.2537             | γ-myb avian myeloblastosis viral oncogene homolog-like 1 | 8.970013 | 0.001        |
| 2060528                | Hs.75227            | NADH dehydrogenase (ubiquinone) 1α subcomplex, 9 (39 kDa) | 8.7508083 | 0.02         |
| 1375235                | Hs.1019             | Parathyroid hormone receptor 1 | 8.5844637 | 0.005        |
| 1419396                | Hs.68583            | Mitochondrial intermediate peptidase | 8.3663583 | 0.0025       |
| 1927914                | Hs.278              | Pre-T/NK cell-associated protein | 8.0456544 | 0.001        |
| 2459178                | Hs.159205           | Basic leucine zipper nuclear factor 1 (JEM-1) | 7.8532877 | 0.005        |
| 2868138                | Hs.111334           | Ferritin, light polypeptide | 7.4725041 | 0.0025       |
| 2823937                | Hs.274614           | Sulfotransferase family, cytosolic, 1A, phenol-prefering, member 3 | 7.4138369 | 0.01         |
| 4060668                | Hs.19492            | Protocadherin 8 | 7.0289136 | 0.014        |
| 1904453                | Hs.159492           | Spastic ataxia of Charlevoix-Saguenay (sacsin) | 6.0857279 | 0.001        |
| 638194                 | Hs.169172           | Calpain 6 | 6.0190133 | 0.005        |
| 4507259                | Hs.144901           | Nuclear receptor co-repressor 1 | 5.966935 | 0.005        |
| 2406795                | Hs.32963            | Cadherin 6, K-cadherin (fetal kidney) | 5.1241682 | 0.005        |
| 2132231                | Hs.89560            | Iduronidase, α-1 | 5.0378157 | 0.025        |
| 1653686                | Hs.75087            | Fas-activated serine/threonine kinase | 4.985012 | <0.001       |
| 5015851                | Hs.78869            | Transcription elongation factor A (SH1), 1 | 4.9720183 | 0.05         |
| 4707270                | Hs.91343            | γ-Aminobutyric acid (GABA) A receptor, α 2 | 4.7696056 | 0.001        |
| 671555                 | Hs.74649            | Cytochrome c oxidase subunit Vc | 4.7407836 | <0.001       |
| 1291022                | Hs.100641           | Caspase 9, apoptosis-related cysteine protease | 4.4978475 | 0.005        |
| 2308184                | Hs.158300           | Huntington-associated protein 1 (neuron 1) | 3.9725666 | 0.045        |
| 2044961                | Hs.3850             | Homo sapiens clone 23956 mRNA sequence | 3.8313785 | 0.05         |
| 2254491                | Hs.26510            | Vacuolar protein sorting 33B (yeast homolog) | 3.7674475 | 0.005        |
| 1700340                | Hs.12109            | WD40 protein Ciao1 | 3.5175671 | 0.016        |
| 1805712                | Hs.54649            | Putative nucleic acid binding protein 1Y-1 | 3.5071528 | 0.029        |
| 2221768                | Hs.5501             | KIAA1194 protein | 3.4896893 | 0.01         |
| 4920069                | Hs.150917           | Catenin (cadherin-associated protein), α 2 | 3.4415667 | 0.005        |
| 556290                 | Hs.128316           | Apoptosis-associated tyrosine kinase | 3.4284854 | 0.069        |
| 2861523                | Hs.155419           | BCL2-interacting killer (apoptosis-inducing) | 3.1179559 | 0.014        |
| 1001970                | Hs.6241             | Phosphoinositide 3-kinase, regulatory (p85 alpha), Fik3r1 | 2.025 | 0.02         |
Pi3k Regulates the Postoperative Apoptotic Phenotype

between primary and recurrent tumor epithelia. The hybridization signal for the gene encoding p85α (i.e. Pik3r1) was significantly reduced within recurrent tumor epithelia (MSD = 2.025, p = 0.02). The signal for the gene encoding the p110 catalytic subunit (i.e. Pik3CA) was significantly increased in expression (MSD = 0.365, p = 0.045). Although numerous other gene changes were observed, these occurred in isolation in that they were not associated with changes in other components within their associated molecular complex. In other words, Pi3k was the only complex in which a collective change occurred for its component subunits. These changes were marginally significant, however, they were consistent and were further confirmed at the protein level (Fig. 5A).

Furthermore, the differential regulation of inhibitory and catalytic Pi3k subunits was associated with a pronounced increase in Akt phosphorylation within recurrent tumors (Fig. 5A). This occurred despite no overt changes in whole cell Akt levels between primary and recurrent tumors (Fig. 5A).

In Vivo and ex Vivo Pi3K Blockade Attenuates the Chemoresistant/Antiapoptotic Phenotype Apparent in Recurrent Tumors—Pi3k has previously been implicated in wound healing, inflammation, angiogenesis, tumorigenesis, and enhanced metastasis formation (31–34). In particular, Pi3k has been strongly associated with enhanced resistance to chemotherapeutic agents such as TRAIL (35–37). Based on this it was proposed that Pi3K played a role in generating the antiapoptotic and chemoresistant phenotype in recurrent tumors.

As described above, recurrent tumor epithelial isolates were more resistant to the effects of TRAIL, than were primary tumor epithelia. To characterize a potential role for Pi3K in leading to this enhanced resistance, purified recurrent tumor cells were co-incubated with LY294002 and TRAIL. Primary tumor epithelia were used as a control. Incubation with LY249002 did not significantly increase apoptosis in primary epithelial cells. TRAIL increased apoptosis in primary tumor epithelia, but apoptosis levels remained unaltered with the addition of LY294002 (p > 0.05). No increases in apoptosis were observed when recurrent tumor epithelia were incubated with LY249002 alone (p = 0.3, Fig. 5B). Although, in recurrent tumor epithelia, incubation with TRAIL led to an increase in apoptosis, co-incubation with TRAIL and LY294002 significantly enhanced this effect (p < 0.001, Fig. 5B). Hence, the targeting of Pi3K further sensitized recurrent tumor epithelium to the proapoptotic effects of TRAIL.

To determine the effects of Pi3K in vivo, LY294002 was administered intraperitoneally for 7 days post tumor cytoreduction. As can be seen, recurrent tumor growth occurred rapidly following cytoreduction (group E, Fig. 6A). Within 10 days of cytoreduction, the volume of recurrent tumors was similar to that of unoperated primary tumors (data not shown). Growth curves for primary and recurrent tumors were markedly different. However, when LY294002 was administered immediately postoperatively, recurrent tumor growth slowed markedly to less than that of the corresponding primary tumor during its initial growth phase (†, p < 0.001; ‡, p < 0.001; and Y, p < 0.001, Fig. 6A). Neither the rate nor the shape of the primary tumor growth curve were altered by the administration of LY294002.

The benefits of LY294002 administration also translated into improved survival (Fig. 6B). Animals that received LY294002 during the immediate postoperative interval had significantly improved survival at and prior to 32 days postoperatively (†, p < 0.001; ‡, p < 0.001; and Y, p < 0.001; Fig. 6B). When reviewed histologically, LY294002-treated tumor recurrences more closely resembled primary tumors with similar levels of TUNEL-positive cells apparent (†, p = 0.3, Fig. 6C). This correlated with a significant reduction in Akt phosphorylation (Fig. 6D, lane 3) and occurred in the absence of any overt dermal toxicity. Of note, whole cell Akt levels did not differ significantly between primary and recurrent tumors (Fig. 6D).

DISCUSSION

Recently, several trials have revisited the concept that tumor removal, and the processes by which this is achieved, exert potentiation effects on residual neoplastic tissue (3, 7). This is not a novel concept, and has origins in a number of historic observations (1, 38). Paget noted that patients who underwent operative treatment of breast cancer often fared worse than those managed conservatively (39). Accelerated postoperative tumor growth has been repeatedly associated with reduced cell apoptosis (1, 2, 4, 5, 14, 17, 18, 20, 22–24, 26, 40–42). However, there is a complete lack of data outlining the intracellular mechanisms that underpin this association. Herein, we firstly demonstrated that local tumor recurrences that arose after cytoreduction, did so in an accelerated manner associated with reduced levels of apoptosis. In the clinical setting, cytoreductive surgery has frequently, though not always, been associated with accelerated recurrent tumor growth (13, 43–45). In the present study, accelerated recurrence occurred irrespective of the origin of the cell line used, was not effected by the immune status of the animals used, and occurred for explanted
FIG. 6. Ten animals were included in each of the groups described below. A, tumor volumes in animals undergoing subtotal excision with postoperative Me₂SO (group E) and with postoperative LY294002 (group F). In the absence of intraperitoneal LY294002, recurrent tumors developed at a markedly accelerated rate relative to corresponding primary tumors. When LY294002 was administered postoperatively recurrent tumor growth rates were significantly reduced (†, p < 0.001; ‡, p < 0.001; and ⋄, p < 0.001; all t test). B, postoperative survival for animals that underwent cytoreduction but did not receive LY294002 (group A), for those undergoing cytoreduction and receiving Me₂SO (group B), and for those that underwent cytoreduction but received LY294004 postoperatively (group C) (†, p < 0.001; ‡, p < 0.001; and ⋄, p < 0.001). C, levels of apoptosis as indicated by TUNEL staining for groups (group A control animals received no treatment, group B received intraperitoneally Me₂SO, group C received intraperitoneal LY294002, group D underwent subtotal tumor excision with no adjuvant treatment, group E underwent cytoreduction and received Me₂SO postoperatively, and group F underwent surgery and received LY294002 postoperatively) (†, p = 0.3; ‡, p < 0.001; t test). D, whole cell Akt and phospho-Akt phosphorylation in primary and corresponding recurrent tumors. Akt phosphorylation in untreated (i.e. group A) primary tumors (lane 1), in untreated (i.e. group D) recurrent tumors (lane 2) and in LY294002 treated (i.e. group F) recurrent tumors (lane 3).
human xenografts. Hence, this was a general phenomenon that represented an excellent template to further study accelerated postoperative tumor growth as well as the mechanisms that underpin it.

To establish the basis for this phenomenon, highly purified primary and recurrent tumor epithelial cell fractions were compared. First, it was noted that primary tumors developed faster and metastatic burden increased earlier, in animals inoculated with recurrent tumor epithelial cells. This pointed to enhanced spontaneous tumorigenicity in recurrent tumor epithelia. Such observations are supported by the findings of enhanced tumorogenicity as well as tumor progression in locally recurrent tumors, in the clinical setting (3, 7, 8, 20). Baseline apoptosis was significantly reduced in recurrent tumor epithelial cells. Moreover, when exposed to TRAIL, the increases in apoptosis observed for primary tumor cells were significantly attenuated. These findings pointed to enhanced chemoresistance in recurrent tumor epithelial cells.

To further compare primary and recurrent tumor epithelia, gene expression profiles were used as a platform to screen for transcriptional differences between the two cell populations. This identified an inversion in the expression levels of regulatory and catalytic subunits of class Ia PI3K, Pik3r1, the gene encoding the regulatory subunit p50, which was significantly reduced in terms of expression. By contrast, Pik3CA, the gene encoding the catalytic subunits p110γ, was significantly increased in terms of overall expression. These findings pointed to a collective change in the subunits that comprise the class Ia PI3K molecular complex. They were confirmed at the protein level and, moreover, were associated with increases in Akt phosphorylation within recurrent tumors. Interestingly, a similar subunit phenotype has previously been associated with enhanced cell survival both in vitro and in vivo (46–48).

In the murine model described herein, multiple physiological (i.e. inflammation and wound healing) and pathological (i.e. increased chemoresistance and reduced apoptosis) processes occur simultaneously. Extensive literature indicates that increased PI3K activity is implicated in each of these processes. As such PI3K may represent a critical signaling point whereby PI3K plays a major role in generating the progressive phenotype during the postoperative interval. Importantly, however, this relationship may be further regulated by targeting PI3K with LY294002. As such, this represents a therapeutic entry point whereby patients may be protected against potentially detrimental host/tumor interactions during the postoperative period.

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