Evidence for the Clonal Origin of Spontaneous Metastases

Abstract. *A cultured cell line of the K-1735 melanoma was x-irradiated to induce chromosome breakage and rearrangements and then was implanted into the footpads of syngenic C3H mice. Spontaneous lung metastases were isolated from different animals, established in culture as individual lines, and then karyotyped. Within certain metastases, the same chromosomal abnormality (or abnormalities) (recombinant chromosomes) was found in all the cells examined. Most metastases differed from one another in that they exhibited characteristic combinations of chromosomal markers. These findings indicated that the metastases were clonal and that they probably originated from different progenitor cells.*

The major cause of death from cancer is the progressive growth of metastases that are resistant to therapy. Malignant neoplasms do not have a uniform cellular composition but consist of subpopulations of cells with different biological characteristics, including degrees of metastatic potential (*1*). Metastasis begins with the invasion of blood vessels or lymphatics (or both) by tumor cells that subsequently disseminate to distant organs. These tumor cells can circulate either as multicellular aggregates or as single cells. After metastatic cells are arrested in the capillary bed of an organ, they extravasate into the parenchyma and proliferate into secondary tumor growths (*2*). Metastatic cells, therefore, must progress through a series of potentially lethal steps. Completion of the steps depends on both host factors and intrinsic tumor cell properties (2). Metastases generally do not result from the random survival of cells released from the primary neoplasm (*3*) but from the selective growth of specialized subpopulations of metastatic cells that preexist in the parent tumor (*4*).

Two questions with important implications for the design of cancer therapies are whether metastases are clonal in origin and whether multiple metastases in a host originate from the same progenitor cell. If this is the case, then a treatment modality directed against one metastasis is likely to be effective against all other secondary growths as well. Alternatively, if different cancer metastases originate from different metastatic progenitor cells, then their response to therapy is likely to be heterogeneous.

To determine whether spontaneous metastases originate from different progenitor cells, we performed an experiment fashioned after the study of Becker *et al.* (*5*), who demonstrated that the pluripotentiality of bone marrow cells that proliferate into spleen colonies in x-irradiated mice. We used the K-1735-M2 tumor line isolated from the K-1735 melanoma, a tumor recently induced in a C3H mouse (*6*). Karyotypic analysis of the K-1735-M2 tumor growing in culture or in a subcutaneous site or in metastases revealed that all of its chromosomes are telocentric. Tumor cells growing in culture were therefore treated with 650 roentgens of x-irradiation to induce chromosomal abnormalities (*5*). The x-irradiated K-1735-M2 cells (10^5) were injected into the footpads of C3H mice. When tumors reached a diameter of 1.2 to 1.5 cm, the tumor-bearing leg, including the involved popliteal lymph node, was amputated. The mice were killed 6 weeks later, and the lungs bearing metastases were removed aseptically. Only lungs containing fewer than five well-isolated metastases, each ranging in size from 2 to 4 mm in diameter, were used. A total of 21 individual spontaneous pulmonary metastases were recovered from 18 different animals and established in culture as individual cell lines (*7*). Chromosome analysis was performed on at least 100 spreads of each individual line with the use of conventional techniques (*8*). X-irradiation is known to induce random chromosome breaks and recombinations (*5*). Therefore, if a spontaneous metastasis originated from a single marked progenitor cell, all the tumor cells within the metastatic focus should express the same chromosomal abnormality.

Table 1 lists the karyotypic analyses of the parent K-1735-M2 line (prior to x-irradiation) and of the 21 lines established from the spontaneous metastases. The nonirradiated parent tumor has a fairly stable mode at 44 chromosomes, all of which are telocentric (Fig. 1a). The modal chromosome numbers of the 21 lines established from metastases ranged from 39 to 46. In 10 of 21 lines, all chromosomes were telocentric and, therefore, these metastases were noninformative by our criteria. Single or multiple marker chromosomes (submetacentric, metacentric, minute) were observed in the following 11 lines: x-met-9, x-met-10, x-met-11, x-met-12 (Fig. 1b), x-met-13, x-met-14, x-met-16 (Fig. 1c), and x-met-21 (Fig. 1d), x-met-5, x-met-17 and x-met-7. Characteristic patterns of chromosomal markers were found in most

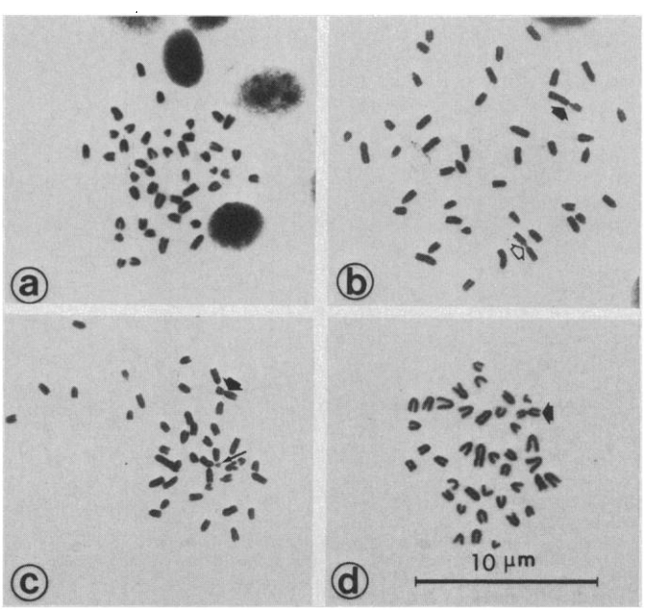

Fig. 1. Metaphase spreads of parent K-1735-M2 (a), x-met-12 (b), x-met-16 (c), and x-met-21 (d). The 44 chromosomes in the parent cells (a) are all telocentric. The x-met-12 (b) contains two marker chromosomes, both of which are longer than the longest telocentric. The submetacentric (➡) is the longest chromosome in the cell and has a ratio of short arm to long arm (p/q) of less than 1:2 with a distinctive secondary constriction on the p arm. The p/q ratio of the secondary marker (⇒) is close to 1:1. The markers of x-met-16 (c) are a submetacentric chromosome (➡) shorter than the longest telocentric (p/q, 1:2) and a very small telocentric (centromeric minute) chromosome (→). Both markers were seen in every cell examined. A single near-metacentric marker (➡) with a ratio of p to q of approximately 3:4 is characteristic of x-met-21 (d).

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spreads from 8 of the 11 lines (Table 1), suggesting that each of these metastases originated from a different progenitor cell. The remaining three lines, x-met-5, x-met-17, and x-met-7, also exhibited centric fusion chromosomes, but in far smaller proportions of the cell populations, suggesting a bimodal or multimodal origin. Many cells were aneuploid or tetraploid, and chromosomal breaks were observed frequently. The marked karyotypic instability, both numerical and structural (*9*), of these three lines obscures the determination of whether they have originated from a single or multiple progenitor cells.

In several lines there were similar chromosome markers. A large metacentric and a large submetacentric chromosome found in cells from x-met-9, x-met-10, and x-met-11 were shown to be identical by G-banding (*10*). These three metastases, therefore, could well have been the progeny of a common progenitor growing in the primary tumor. Similarities between markers in several of the remaining metastases warrant further study. It is possible that x-irradiation or intrinsic factors in the tumor cells promote fusion preferentially between specific chromosomes, although there is little evidence of preferential fusion in spontaneous robertsonian translocations (*11*). Alternatively, certain randomly formed chromosomal translocations could confer a selective advantage upon cells that allow them to complete the metastatic process. Either possibility could account for the parallel evolution of karyotypic markers.

Our results suggest that at least some metastases are clonal in their origin. Analysis of the distribution and fate of circulating tumor emboli has demonstrated that multicellular aggregates (homotypic or heterotypic) are more likely to give rise to a metastasis than a single tumor cell embolus (*12*). The results suggest either that metastases result from the proliferation of a single viable cell within a heterogeneous embolus or that a circulating tumor embolus is likely to be homogeneous because it originated from a clonal zone of a primary neoplasm (*13*). Our data show that, although metastasis is a highly selective process, different metastases can originate from different progenitor cells. This finding accounts for the biological heterogeneity that exists among various metastases (*14*). Moreover, the recent findings that isolated clonal populations tend to be phenotypically unstable (*15*) and that metastatic cells mutate spontaneously more often than nonmetastatic cells (*16*) provide an explanation for the mechanism (or mechanisms) that generates diversity within a metastasis. In any event, the finding that different metastases can originate from different progenitor cells reaffirms the conclusion that the successful approach to the treatment of metastases will circumvent the problem of tumor cell heterogeneity (*17*).

Table 1. Karyotypic analysis of K-1735-M2 parent line and 21 spontaneous lung metastases. The karyotypic analysis of K-1735-M2 parent was performed before x-irradiation. X-met refers to individual spontaneous lung metastases established as cell lines in culture. At least 100 consecutive karyotypes were analyzed per cell line.

Source of cells	Karyotypes					Marker chromosomes (% cells)	
	Chromosome mode	Chromosome range	At mode cells (% ± 3)	Aneuploid* (%) cells	Tetraploid* (%) cells	Metacentric (M)†	Submetacentric‡
K-1735-M2	44	33–50	87	0	0	0	0
X-met-1	45	32–47	83	0	0	0	0
X-met-2	43	32–89	53	14	16	0	0
X-met-3	42	31–54	70	6	21	0	0
X-met-4	43	31–48	78	0	9	0	0
X-met-6	42	38–85	77	0	21	0	0
X-met-8	42	31–92	46	16	12	0	0
X-met-15	44	32–45	80	0	0	0	0
X-met-18	44	31–47	86	0	0	0	0
X-met-19	44	31–46	75	0	0	0	0
X-met-20	41	32–47	80	0	0	0	0
X-met-9	42	37–48	89	0	0	M-1(100)M-2(50)	1:3(80)
X-met-10	40	36–43	98	0	0	M-1(90)	1:3(100)
X-met-11	42	36–43	90	0	0	M-1(60)M-2(20)	1:3(100)
X-met-12	41	36–44	94	0	0	Small M-1(100)	1:2(100)
X-met-13	41	34–47	87	0	3	M-1(90)	1:3(90)
X-met-14	42	39–47	93	0	0	M-1(100)	1:3(95)
X-met-16	42	32–46	83	0	0	0	1:2(100)
X-met-21	42	31–45	73	0	0	0	3:4(100)
X-met-5	42	35–47	76	7	10	M-1(15)	1:3(57)
X-met-17	44	31–45	81	0	0	0	1:3(50)
X-met-7	39	35–148	55	23	8	Variable	Variable

*Aneuploid, karyotypes with >50 chromosomes. Tetraploid indicates karyotypes with 80 to 90 chromosomes. †Number in parentheses refers to the percentage of karyotypes exhibiting the particular chromosome marker. ‡Arm length ratio. Number in parentheses refers to the percentage of karyotypes exhibiting the particular chromosome marker.

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Inorganic Pyrophosphate: Energy Source for Sulfate-Reducing Bacteria of the Genus *Desulfotomaculum*

Abstract. *Sulfate-reducing bacteria belonging to the genus* Desulfotomaculum *utilized inorganic pyrophosphate as a source of energy for growth in the presence of fixed carbon (acetate and yeast extract) and sulfate. Pyrophosphate does not support the growth of* Desulfovibrio *under the same growth conditions. Over a limited range of concentrations, growth is proportional to pyrophosphate, and extracts of bacteria grown on pyrophosphate medium have enzymatic activities similar to extracts prepared from bacteria grown on medium containing lactate plus sulfate. The variety of cell types observed in crude anaerobic pyrophosphate-enrichment cultures from a marine environment suggests that this unique type of energy metabolism is not restricted to the sulfate-reducing bacteria of the genus* Desulfotomaculum.

Inorganic pyrophosphate (PP_i) has been proposed (*1*) as an evolutionary precursor of adenosine triphosphate (ATP) and more recently has been shown to be involved in a number of energy-yielding reactions (*2*). Liu and Peck (*3*) demonstrated that the bioenergetics of respiratory sulfate reduction by two of the described genera of sulfate-reducing bacteria, *Desulfovibrio* and *Desulfotomaculum*, are fundamentally different. In the case of *Desulfovibrio*, the PP_i produced during the formation of adenylyl sulfate (APS) from ATP and sulfate by ATP-sulfurylase (Eq. 1)

$$ATP + SO_4^{2-} \rightarrow APS + PP_i \quad (1)$$

in the first enzymatic step of respiratory sulfate reduction is hydrolyzed to orthophosphate (P_i) by inorganic pyrophosphatase (Eq. 2)

$$PP_i + H_2O \rightarrow 2P_i \quad (2)$$

Thus, the chemical energy in the anhydride bond of PP_i is not conserved and, in order to obtain a net yield of ATP during growth on lactate plus sulfate, *Desulfovibrio* must carry out electron transfer–coupled phosphorylation. In contrast, *Desulfotomaculum* is able to conserve the bond energy of the pyrophosphate produced by ATP-sulfurylase (Eq. 1) by means of the enzyme acetate: PP_i phosphotransferase (Eq. 3) (*4*).

$$\text{Acetate} + PP_i \rightarrow \text{acetyl phosphate} + P_i \quad (3)$$

Adenosine triphosphate can then be produced from acetyl phosphate and adenosine diphosphate (ADP) by acetate kinase (Eq. 4).

$$ADP + \text{acetyl phosphate} \rightarrow \text{acetate} + ATP \quad (4)$$

These two enzymatic reactions allow *Desulfotomaculum* to generate one high-energy phosphate by substrate-level phosphorylation per sulfate reduced to sulfide during growth with lactate, and it is not necessary for this microorganism to carry out electron transfer–coupled phosphorylation during growth with lactate and sulfate. These observations suggested the possibility that *Desulfotomaculum* might be capable of utilizing PP_i as a source of ATP for growth in the presence of fixed carbon. In this report, we describe the growth of *D. nigrificans*, *D. ruminis*, and *D. orientis* on a medium containing PP_i, acetate, yeast extract, sulfate, and salts. The results obtained with crude enrichment cultures from a marine spartina marsh suggest that PP_i respiration is a more general phenomenon among anaerobic microorganisms and is not restricted to the genus *Desulfotomaculum*.

The conditions for the anaerobic growth of *D. nigrificans* on PP_i are given in Table 1. The basal medium, containing acetate, yeast extract, sulfate, and salts, does not support growth of this bacterium. However, when the basal medium is supplemented with PP_i, growth is better than that obtained under usual growth conditions with lactate plus sulfate. On the basal medium, PP_i does not stimulate the growth of *Desulfovibrio vulgaris*, and

Table 1. Requirements for the growth of *Desulfotomaculum nigrificans*, with inorganic pyrophosphate as a source of energy. Growth was measured, in optical density (O.D.) units, at 580 nm in 1-cm cuvettes and expressed as averages of duplicate flasks (500 ml, containing 200 ml of medium) after 48 hours of incubation at 55°C under argon. A 5 percent inoculum of bacteria grown on the PP_i medium was used in all experiments. The basal medium contained per liter: sodium acetate, 3.3 g; Na_2SO_4, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MgCl_2 \cdot 6H_2O$, 1.8 g; K_2HPO_4, 0.5 g; $CaCl \cdot 2H_2O$, 0.2 g; Difco yeast extract, 2.0 g; $FeSO_4$, 10 mg; reducing agent (2.5 g of cysteine · HCl plus 2.5 g of $Na_2S \cdot 9H_2O$ per 200 ml of H_2O), 20 ml. KOH was used to adjust the *p*H to 7.2. Where indicated, the 0.05 percent PP_i (filter sterilized) was added. The lactate-sulfate medium contained per liter: sodium lactate (60 percent), 12.5 ml; NH_4Cl, 2.0 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; K_2HPO_4, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.2 g; Difco yeast extract, 1.0 g; and $Na_2S \cdot 9H_2O$, 0.25 g.

Additions and deletions	Growth (O.D.)
Basal medium	0.019
Plus PP_i	0.628
Plus PP_i; minus SO_4^{2-}	0.019
Plus PP_i; minus acetate	0.095
Plus PP_i; minus yeast extract	0.042
Plus PP_i; minus acetate and yeast extract	0.036
Lactate-sulfate medium	0.505

Table 2. Enzymatic activities in extracts of *Desulfotomaculum orientis* grown on either pyrophosphate medium or lactate-sulfate medium. For assay procedures, see text. Abbreviations: MV, methyl viologen; BV, benzyl viologen.

Enzymatic activity	Specific activity (nmole/min per mg protein)	
	Lactate-sulfate medium	PP_i medium
Bisulfite reductase ($MV^+ \rightarrow HSO_3^-$)	64.3	57.1
Nitrite reductase ($MV^+ \rightarrow NO_2^-$)	119.6	153.8
Thiosulfate reductase ($MV^+ \rightarrow S_2O_3^{2-}$)	23.7	21.5
Fumarate reductase ($MV^+ \rightarrow$ fumarate)	0	0
APS reductase [AMP + $SO_3^{2-} \rightarrow Fe(CN)_6^{3-}$]	397	385
Formate dehydrogenase (formate $\rightarrow BV^{2+}$)	64	87.1
Hydrogenase ($H_2 \rightarrow BV^{2+}$)	16.2	85.2
Pyruvate dehydrogenase (pyruvate $\rightarrow BV^{2+}$)	107	139
ATP-sulfurylase	140	151
PP_i:acetate kinase	820	1315
Inorganic pyrophosphatase	149	98

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