

Myogenic cell proliferation and generation of a reversible tumorigenic phenotype are triggered by preirradiation of the recipient site

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Environmental influences have profound yet reversible effects on the behavior of resident cells. Earlier data have indicated that the amount of muscle formed from implanted myogenic cells is greatly augmented by prior irradiation (18 Gy) of the host mouse muscle. Here we confirm this phenomenon, showing that it varies between host mouse strains. However, it is unclear whether it is due to secretion of proliferative factors or reduction of anti-proliferative agents. To investigate this further, we have exploited the observation that the immortal myogenic C2 C12 cell line forms tumors far more rapidly in irradiated than in nonirradiated host muscle. We show that the effect of

preirradiation on tumor formation is persistent and dose dependent. However, C2 C12 cells are not irreversibly compelled to form undifferentiated tumor cells by the irradiated muscle environment and are still capable of forming large amounts of muscle when reimplanted into a nonirradiated muscle. In a clonal analysis of this effect, we discovered that C2 C12 cells have a bimodal propensity to form tumors; some clones form no tumors even after extensive periods in irradiated graft sites, whereas others rapidly form extensive tumors. This illustrates the subtle interplay between the phenotype of implanted cells and the factors in the muscle environment.

Introduction

Developmental biology portrays a complex model of control of cell movement and differentiation on the basis of cell signaling mechanisms specified within a sequence of local environments. But little is known about the equivalent processes that maintain tissue integrity in the adult, where it is particularly relevant to the accurate reconstruction and regeneration that ensues after injury of most tissues. This process involves coordinated activation, proliferation, migration, and differentiation of the component precursor cells

to form new tissue. Neoplasia represents uncontrolled manifestation of one or more of these activities.

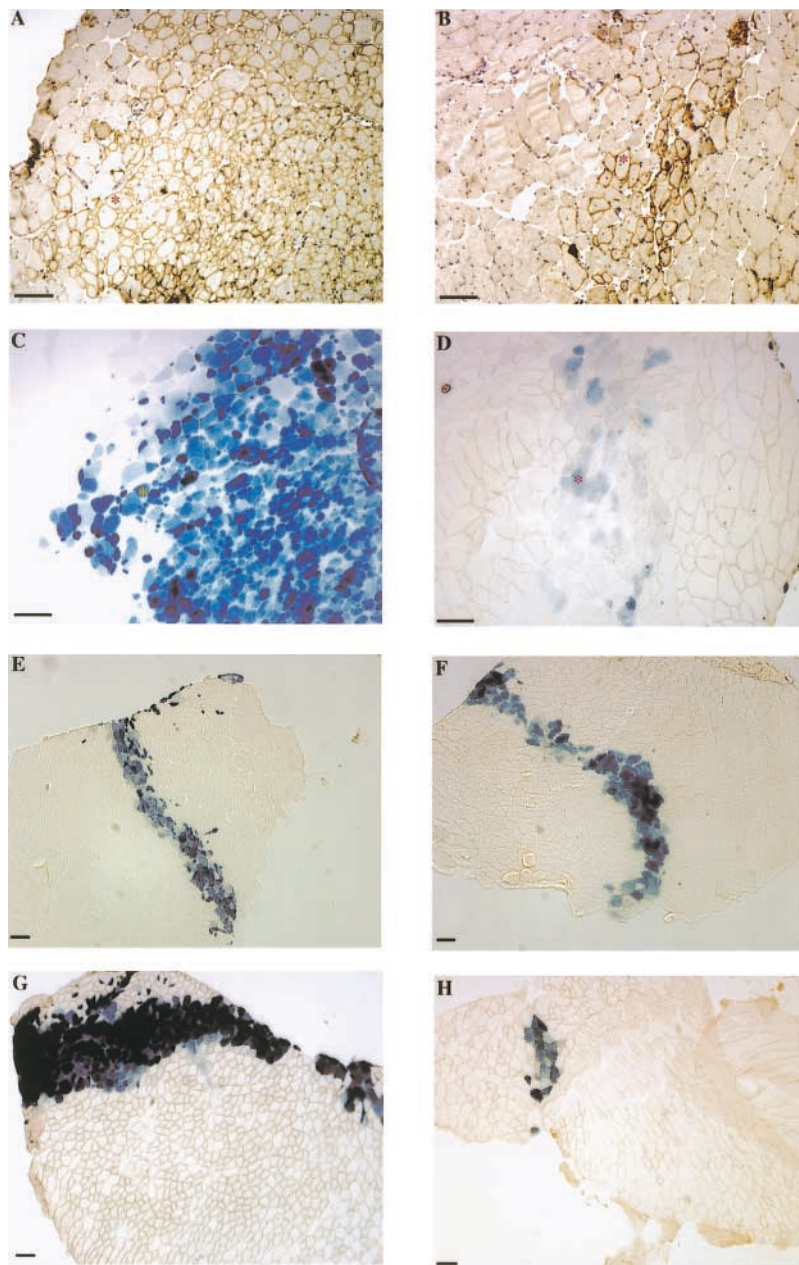
Skeletal muscle regeneration is a well-studied model that superficially closely recapitulates embryonic myogenesis in which activation, proliferation, and subsequent differentiation of muscle precursor cells (MPC)* to form new muscle tissue has been well described both in tissue culture and in vivo. We have attempted to bridge the gap between in vivo and in vitro models by transplantation of myoblasts, using the immunodeficient, dystrophin-deficient mdx nu/nu mouse host. A major goal in this work is to identify environmental factors that optimize muscle formation from implanted cells. Thus far, the most effective procedure is preirradiation of host mouse muscle with 18 Gy. This causes the implanted

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*Abbreviations used in this paper: FGF, fibroblast growth factor; MMP, matrix metalloproteinase; MPC, muscle precursor cell; TA, tibialis anterior.

Figure 1. Transverse cryostat sections of TA muscles that had been injected with H2K 18.30 MPC 35 d earlier. (A) Muscle from the irradiated right leg of an mdx nu/nu mouse, stained with antidystrophin antibody and counterstained with haematoxylin to show the nuclei. A large group of dystrophin-positive fibers is shown. (B) Muscle from the contralateral, nonirradiated leg of an mdx nu/nu mouse, stained with antidystrophin antibody and counterstained with haematoxylin to show the nuclei. A few dystrophin-positive fibers are shown. (C) Serial section to A, stained for β -gal. A large group of donor fibers is present within this muscle. An asterisk marks the same fiber in sections A and C. (D) Serial section to B, stained for β -gal. Again, only a few donor fibers are found in the muscle. An asterisk marks the same fiber in sections B and D. (E) Muscle from the irradiated right leg of a beige/nu/Xid mouse, stained for β -gal. A small number of donor fibers are present only along the injection site. (F) Muscle from the nonirradiated left leg of a beige/nu/Xid mouse, stained for β -gal. As in E, a small number of donor fibers are present only along the injection site. (G) Muscle from the irradiated leg of a $C5^{-/\gamma}$ chain-deficient/Rag2⁻ mouse, stained for β -gal. A large group of donor muscle fibers is present within this muscle. (H) Muscle from the nonirradiated left leg, contralateral to the muscle shown in G. Only a very few β -gal-positive fibers are present. Bars, 100 μ m.



cells to proliferate (Beauchamp et al., 1999), form more muscle, and migrate to contiguous muscles more frequently than in the nonirradiated leg (Morgan et al., 1993). The irradiated muscle environment is thus clearly beneficial for myoblast transplantation. However, the dose is too high to be considered as a therapeutic option and we need, therefore, to understand its mechanism of action in the hope of reproducing it by less extreme means.

To this end, we have established a simple assay for the effect of preirradiation of the host muscle on implanted MPC, using the myogenic cell line C2 C12 (Yaffe and Saxel, 1977; Blau et al., 1983). These cells form muscle upon implantation into mouse muscle, but eventually form tumors (Wernig et al., 1991; Morgan et al., 1992). Such tumors formed far more rapidly in irradiated than in nonirradiated mdx nu/nu mouse muscles (Pagel et al., 2000), thus constituting a rapid and sensitive assay for the growth promoting effects of irradiation.

Here, we have used C2 C12-derived tumor formation as a measure of radiation-induced stimulation of muscle cell proliferation, showing that this effect is persistent and that switching of the C2 C12 cell phenotype from myogenic differentiation to aggressive neoplastic behavior is reversible. We have also identified individual retrovirally marked subclones of C2 C12 cells that do or do not show this capacity for conversion between neoplasia and differentiation.

Results

Implanted H2K 18 myoblasts form more muscle in irradiated muscles in some host strains

Significantly more muscle of donor origin was formed in irradiated mdx nu/nu and $C5^{-/\gamma}$ chain-deficient/Rag2⁻ tibialis anterior (TA) muscles than in contralateral, nonirradiated muscles. This was true whether the number of dystrophin- or

Table I. Muscle formation from H2K 18.30 cells in irradiated and nonirradiated host muscles

| Experiment | Radiation dose | Host mouse | No. of mice | Dystrophin-positive fibers | β -Gal-positive fibers | Male DNA | β -Gal activity |
|------------|----------------|-----------------|-------------|----------------------------|------------------------------|---------------------------|-----------------------|
| A1 | 18 | mdx nu | 6 | 263.8 (57.0) ^a | 247.0 (55.8) ^a | | |
| A1 | 0 | mdx nu | 6 | 78.7 (19.4) | 80.5 (18.5) | | |
| A2 | 18 | mdx nu | 7 | 601.6 (116.4) ^a | 628.3 (119.7) ^{a,b} | | |
| A2 | 0 | mdx nu | 7 | 209.6 (43.5) | 173.1 (32.6) | | |
| B | 0 | mdx nu | 6 | 196.0 (34.4) | 173.3 (36.4) | | |
| C | 18 | mdx nu | 6 | | | 249.8 (53.8) ^a | |
| C | 0 | mdx nu | 6 | | | 71.3 (8.8) | |
| D | 18 | mdx nu | 7 | | | | 1.02 (0.28) |
| D | 0 | mdx nu | 7 | | | | 0.3 (0.1) |
| E | 18 | Bnx | 6 | | 145.2 (33.2) | | |
| E | 0 | Bnx | 6 | | 104.5 (33.8) | | |
| F | 0 | Bnx | 6 | | 122.2 (21.7) | | |
| G | 18 | C5 ⁻ | 6 | | 188.3 (19.8) ^a | | |
| G | 0 | C5 ⁻ | 6 | | 19.2 (11.9) | | |
| H | 0 | C5 ⁻ | 4 | | 99.4 (41.1) | | |

Conditionally immortal H2K 18.30 MPCs were implanted into irradiated and nonirradiated TA muscles of mdx/nu/nu (mdx nu), beige/nu/Xid (Bnx), C5⁻/gamma chain-deficient/Rag2⁻ (C5⁻) host mice. In three experiments (experiments B, F, and H), the values given are for both the left and right legs. The amount of male DNA is given as ng/muscle. The amount of β -gal activity is U/muscle. The radiation dose is given as grays (Gy). The values in the last four columns are the mean with the SEM in parentheses.

^aStatistically significant difference (Mann-Whitney test) between values for the irradiated right TA and nonirradiated left TA.

^bStatistically significant difference between experiments 1 and 2 (Mann-Whitney test).

β -gal-positive fibers or the amount of Y chromosome was used as the measure of donor muscle in mdx nu/nu hosts (Fig. 1; Table I). However, mean activity of extracted β -gal was not significantly greater in homogenates prepared from irradiated than nonirradiated mdx nu/nu muscles, indicating that this may not be a reliable method for determining donor contribution to grafted muscle (Table I). Importantly, the number of dystrophin- and β -gal-positive fibers in representative sections of mdx nu/nu muscles showed a 1:1 concurrence, the slope of the number of β -gal-positive fibers against the number of dystrophin-positive fibers was 1.01 ($r^2 = 0.93$; $n = 38$), so we could confidently use β -gal immunostaining in nonmyopathic hosts to quantify the amount of donor muscle formed.

However, when the same cells were implanted into nonmyopathic beige/nu/Xid mouse muscles, the amount of donor muscle was not significantly greater in irradiated than in nonirradiated muscles. (Fig. 1; Table I).

Our results show that radiation delivered to one leg does not have a growth-promoting effect on cells implanted into the contra-lateral leg. There was no significant difference in the number of donor muscle fibers between the nonirradiated muscles of mice that had their right leg irradiated (Table I, experiments A2, E, and G) and mice of the same strain that had neither leg irradiated (Table I, experiments B, F, and H).

Preirradiation of host muscle augments C2 C12-derived tumor formation

In both mdx and nonmyopathic immunodeficient host strains, implantation of 5×10^5 C2 C12 cells leads to the formation of macroscopically visible tumors in all of the irradiated muscles, but in none of the nonirradiated muscles (Fig. 2). Histologically, irradiated mdx nu/nu TA muscles that had been injected with C2 C12 cells contained large numbers of dystrophin-positive fibers, but also conspicuous areas of undifferentiated interstitial cells, which we presume to be tumor cells (Fig. 3 A). Similar undifferentiated cells were seen in irradiated, C2 C12 cell-injected beige/nu/Xid muscles (unpub-

lished data). Nonirradiated muscles contained smaller numbers of undifferentiated cells, interspersed diffusely between donor muscle fibers (Fig. 3 B). In both host strains, significantly more nondifferentiated interstitial cells were found in the irradiated right TA than in the nonirradiated left TA (Table II, experiments A1, A2, and F). Thus, 18 Gy of gamma radiation, delivered to the host muscle 3 d before cell implantation, augments tumor formation from implanted C2 C12 cells in both myopathic and normal mouse muscles.

Similar effects were seen when nonmuscle cells were implanted into irradiated and nonirradiated mdx nu/nu TA muscles, but these were less striking when nontransformed Swiss 3T3 (donated by Professor Gullick, ICRF, London, UK) rather than NIH 3T3 cells were used (unpublished data), indicating that factors within irradiated muscle are widely involved in cell proliferation.

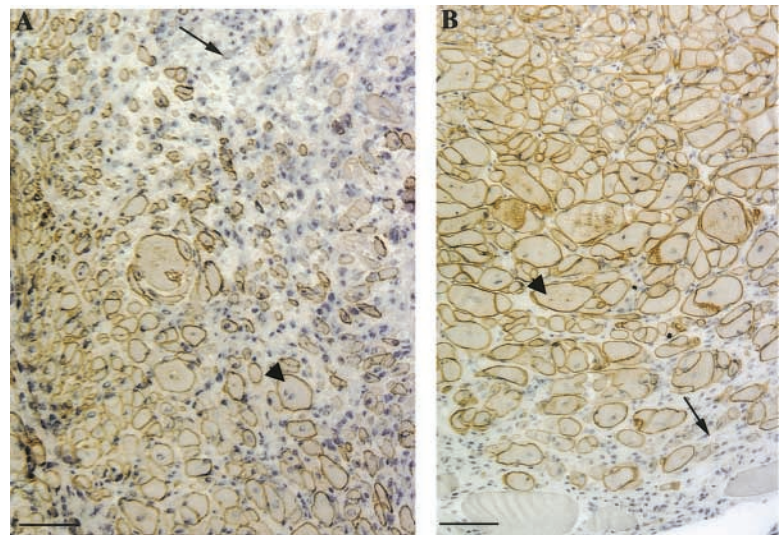
Preirradiation of host skeletal muscle leads to a persistent enhancement of tumorigenesis

Visible tumors were found in all of the irradiated muscles that had been injected with C2 C12 cells at all three time points after irradiation (Table II, experiments A–C). They



Figure 2. Mdx nu/nu mouse, whose right leg was irradiated with 18 Gy 3 d before the injection of 5×10^5 C2 C12 cells into the right and left TA muscles. (A) 2 and (B) 6 wk after cell implantation.

Figure 3. Transverse cryostat sections of TA muscles that had been injected with C2 C12 cells, stained for dystrophin, and counterstained with haematoxylin. (A) Mdx nu/nu TA muscle that had been irradiated with 18 Gy and then injected with C2 C12 cells 3 d later. (B) Muscle from the contralateral, nonirradiated leg. Arrows indicate undifferentiated cells; arrowheads indicate dystrophin-positive fibers. Bars, 100 μ m.



were also seen in 2/7 of the nonirradiated muscles that were contralateral to muscles irradiated 28 d before cell implantation. Histologically, significantly more interstitial cells were found in the irradiated right TA muscles than in the nonirradiated, contralateral muscle at all three time points. Most undifferentiated tissue was found when the cells were injected 28 d after irradiation. However, noticeably less undifferentiated tissue was found in muscles injected 100 d after irradiation (Table II). This may reflect the loss of muscle mass 100 d after irradiation, leaving fewer irradiated host fibers than at earlier times to influence the implanted C2 C12 cells. Nonetheless, enhancement of C2 C12–derived tumor formation clearly persists for at least 100 d after 18 Gy of ir-

radiation. Moreover, significantly more muscle fibers of donor origin were also found in the muscles injected with C2 C12 cells at this time point than in the contralateral, nonirradiated legs (Table II).

C2 C12 tumorigenicity in vivo is dependent on the dose of preirradiation

Tumors were seen in 4/5 of the muscles irradiated with 9 Gy and in only 4/11 of the muscles irradiated with 4.5 Gy (Table II). The amount of nondifferentiated interstitial cells was far less in the muscles preirradiated with either 9 Gy or 4.5 Gy than in the muscles preirradiated with 18 Gy (Table II). These results show that the radiation dose required to elicit rapid tumor for-

Table II. Muscle and tumor formation from C2 C12 cells, or C2 C12–derived tumor, in irradiated and nonirradiated host muscles

| Experiment | Radiation dose | Host mouse | Interval between irradiation and transplant | Tumor visible | Undifferentiated tissue | Dystrophin-positive fibers |
|------------|----------------|------------|---|---------------|-------------------------|----------------------------|
| A1 | 18 | mdx nu | 3 | 7/7 | 42.7 (5.3) ^a | 767 (256) |
| A1 | 0 | mdx nu | 3 | 0/7 | 8.9 (2.3) | 331 (118) |
| A2 | 18 | mdx nu | 3 | 6/6 | 33.8 (5.0) ^a | 2820 (271) ^{a,b} |
| A2 | 0 | mdx nu | 3 | 1/6 | 2.2 (0.71) ^b | 790 (193) |
| B | 18 | mdx nu | 28 | 7/7 | 62.1 (4.4) ^a | 1189 (282) |
| B | 0 | mdx nu | 28 | 2/7 | 7.6 (2.5) | 679 (253) |
| C | 18 | mdx nu | 100 | 7/7 | 22.1 (2.7) ^a | 1132 (108) ^a |
| C | 0 | mdx nu | 100 | 0/7 | 3.07 (0.95) | 434 (346) |
| D | 9 | mdx nu | 3 | 4/5 | 21.0 (6.2) | 777 (281) |
| D | 0 | mdx nu | 3 | 0/5 | 5.0 (1.8) | 538 (255) |
| E1 | 4.5 | mdx nu | 3 | 3/6 | 3.6 (2.1) | 148 (68) ^a |
| E1 | 0 | mdx nu | 3 | 1/6 | 1.5 (0.73) | 870 (251) |
| E2 | 4.5 | mdx nu | 3 | 1/5 | 8.5 (1.5) ^a | 1011 (182) ^b |
| E2 | 0 | mdx nu | 3 | 0/5 | 0.99 (0.24) | 624 (232) |
| F | 18 | Bnx | 3 | 5/5 | 54.2 (3.8) ^a | ND |
| F | 0 | Bnx | 3 | 0/5 | 8.0 (1.4) | ND |
| G | 18 | mdx nu | 3 | 7/7 | 41.9 (6.9) ^a | 1761 (210) ^a |
| G | 0 | mdx nu | 3 | 0/7 | 12.5 (2.9) | 552 (132) |
| H | 0 ^e | mdx nu | 3 | 0/10 | 1.2 (0.16) | 1261 (349) |

C2 C12 cells, or C2 C12–derived tumor (experiment G), were implanted into irradiated and nonirradiated TA muscles of mdx nu/nu (mdx nu) and beige/nu/Xid (Bnx) mice. In one experiment (experiment H), the results given are for both left and right legs. The radiation dose is given as grays (Gy). The values in the column “tumor visible” are the number of legs that had macroscopically visible tumors out of the total number examined. The interval between irradiation and transplant was measured in days. The undifferentiated tissue is presented as a percentage of the area. The values in the last two columns are the mean with the SEM in parentheses.

^aStatistically significant difference (Mann-Whitney test) between values for the irradiated right TA and nonirradiated left TA.

^bStatistically significant difference between experiments 1 and 2 (Mann-Whitney test).

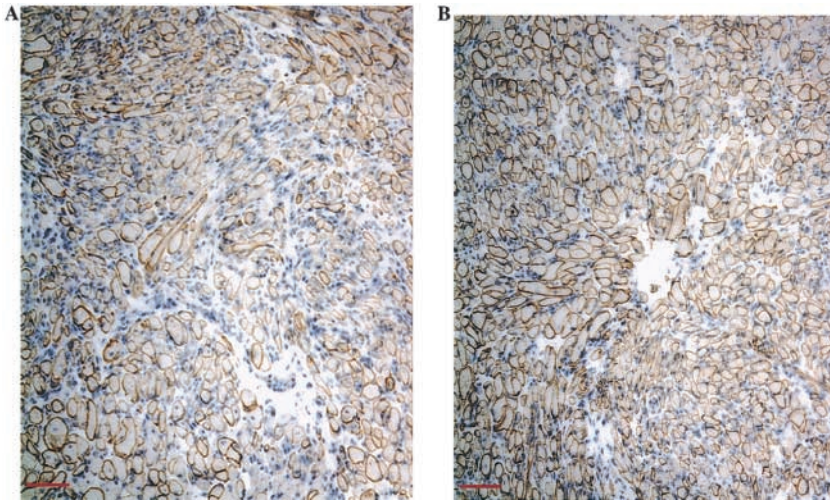


Figure 4. **Transverse cryosections of mdx nu/nu TA muscles implanted with C2 C12–derived tumor, stained for dystrophin, and counterstained with haematoxylin.** (A) TA muscle that had been irradiated with 18 Gy and had C2 C12–derived tumor implanted 3 d after irradiation. (B) Muscle from the non-irradiated leg contralateral to A. Irradiated muscles in this experiment contained more donor muscle fibers than muscles that had been injected with cultured C2 C12 cells (Table II), although the fibers were very small in size (compare Fig. 4 with Fig. 3). Bars, 100 μ m.

mation is critical; 18 Gy is effective, but 9 and 4.5 Gy are both suboptimal to induce C2 C12 cells to rapidly form tumors.

Although there were variations in the amount of tumor and extent of muscle formation between experiments, the results presented in Table II show no significant dose-dependent change in the number of donor muscle fibers in irradiated muscles. There does appear to be more donor muscle in muscles irradiated with 18 Gy compared with contralateral muscles, but this was not always significant. (Table II, experiments A1 and A2).

The finding that the amount of donor muscle and undifferentiated interstitial cells formed in nonirradiated muscles varies from group to group (Table II) seems likely to be due to interexperimental variation, rather than the irradiation of one leg having a systemic or contralateral effect on the opposite, nonirradiated leg. To confirm this, we injected C2 C12 cells from a single batch into both legs of mdx nu/nu hosts that either had their right legs irradiated with 4.5 or 18 Gy or had neither leg irradiated. We found no significant difference in the amount of donor muscle or the amount of non-differentiated tissue in nonirradiated muscles, whether they were from mice in which the right leg had been irradiated with 4.5 or 18 Gy, or from mice which had neither leg irradiated (Table III, experiments A2, E2, and H).

C2 C12 tumors are capable of forming mature muscle upon serial transplant

To examine whether C2 C12–derived tumors are capable of forming muscle in a second recipient host, C2 C12–derived tumor tissue was transplanted from irradiated mdx nu/nu host muscles into irradiated and nonirradiated legs of second recipients, as described in the Materials and methods. 3 wk later, tumors were apparent in the irradiated legs but not in the nonirradiated legs (Table II, experiment G), with irradiated TA muscles containing significantly more undifferentiated interstitial cells than nonirradiated TAs. Moreover, it was noticeable that, in addition to tumors in irradiated sites, both irradiated and nonirradiated muscles contained large numbers of dystrophin-positive donor muscle fibers (Fig. 4), and the amount of donor muscle was significantly greater in irradiated sites.

These results show that implantation of C2 C12–derived tumors into a second series of irradiated or nonirradiated mdx nu/nu mouse muscle leads to the formation of donor muscle, recapitulating the original *in vivo* behavior of C2 C12 cells.

Subclones of C2 C12 cells form tumors

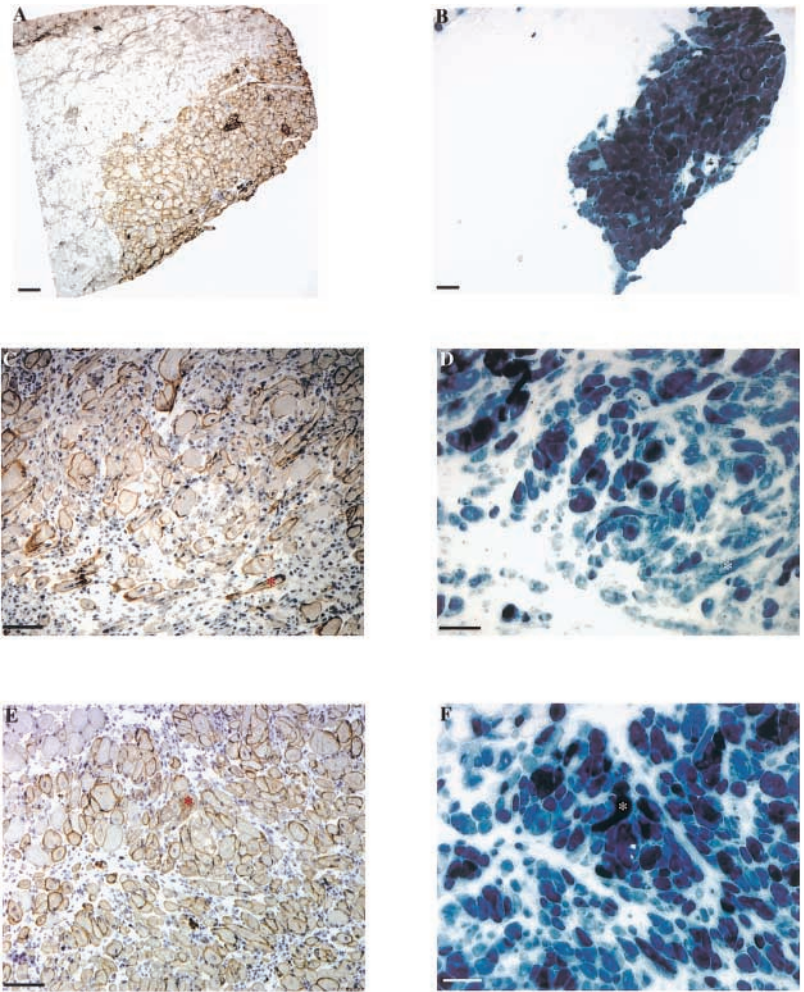
The ability of C2 C12 cells to form tumors and muscle *in vivo* was further examined using subclones derived from a population of C2 C12 cells retrovirally infected with a marker gene and an antibiotic resistance gene. Clones of retrovirally infected C2 C12 cells that were β -gal positive and gave rise to myotubes *in vitro* were isolated and either pooled and analyzed as an oligoclonal population or analyzed as separate clones.

For the oligoclonal analysis, seven clones were coinjected into the irradiated right legs and nonirradiated left legs of 11 mice. Muscles from six of these mice were removed for analysis 21 d after cell injection. No tumors were visible in any of the injected muscles, and sections of these muscles contained very few, if any, undifferentiated cells. The remaining five mice were left until 90 d after grafting; only one of these irradiated muscles contained a small, macroscopically visible tumor. All muscles contained conspicuous amounts of donor muscle (Fig. 5, A and B).

A further four clones were injected separately into irradiated and nonirradiated mdx nu/nu muscles. 3 wk after cell implantation, all clones gave rise to muscle, significantly more donor muscle fibers being found in irradiated than in nonirradiated muscles (Table III; Fig. 5, E and F). None of the clones gave rise to visible tumors in nonirradiated muscles (Table III). Of the four clones examined, clones 2, 9, and 10 gave rise to visible tumors in irradiated legs and formed significantly more undifferentiated interstitial cells in irradiated than in nonirradiated legs. Muscles injected with clone 5 did not develop any visible tumors and contained very little undifferentiated tissue in either irradiated or nonirradiated muscles (Table III; Fig. 5, C and D).

These data show that some subclones of C2 C12 cells form tumors and some do not, indicating a heterogeneity in the original C2 C12 cell line in response to an irradiated muscle environment.

Figure 5. Transverse cryosections of irradiated mdx nu/nu TA muscles injected with C2 C12 cell clones expressing cytoplasmic-localizing LacZ 21 d earlier. (A) Muscle that had been injected with a mixture of seven clones, stained for dystrophin. (B) Serial section to A, stained for β -gal. (C) Muscle that had been injected with clone 2, stained for dystrophin. (D) Serial section to C, stained for β -gal. An asterisk identifies the same fiber in C and D. (E) Muscle that had been injected with clone 5, stained for dystrophin. (F) Serial section to E, stained for β -gal. An asterisk identifies the same fiber in E and F. Bars, 100 μ m.



Candidate myoblast proliferation or migration factors are not augmented in mouse skeletal muscle by radiation

To investigate whether factors that have been implicated in the proliferation or migration of MPCs were altered in irradiated skeletal muscle (Bischoff, 1997; El Fahime et al., 2000; Kastner et al., 2000), we examined the expression of four growth factors (β -fibroblast growth factor [FGF], FGF-4, FGF-6, and hepatocyte growth factor) and two matrix metalloproteinases (MMP-2 and MMP-9) in mdx and C57

Bl/10 muscles 3 d after 18 Gy of irradiation. There were no significant differences in expression of these proteins in irradiated and nonirradiated mdx and C57Bl/10 muscles (Fig. 6). Although the amounts of β -FGF, FGF-4, and FGF-6 were slightly reduced in irradiated C57Bl/10 muscles and slightly elevated in irradiated mdx muscles, these differences were not significant. MMP-2 was slightly elevated in irradiated C57Bl/10 muscles and slightly reduced in irradiated mdx muscles, but there was no change in MMP-9 expression in irradiated muscles.

Table III. Tumor and muscle formation from C2 C12 subclones in irradiated and nonirradiated mdx nu/nu muscles

| Experiment | Radiation dose | Clone implanted | Tumor visible | Undifferentiated tissue | Dystrophin-positive fibers | β -Gal-positive fibers |
|------------|----------------|-----------------|---------------|---------------------------|-----------------------------|------------------------------|
| A | 18 | 2 | 3/7 | 27.61 (3.54) ^a | 727.0 (290.6) ^a | 806.3 (329.8) ^a |
| A | 0 | 2 | 0/7 | 3.00 (1.44) | 130.4 (36.7) | 198.9 (61.7) |
| B | 18 | 5 | 0/5 | 4.95 (1.6) | 494.0 (100.8) ^a | 522.0 (103.3) ^a |
| B | 0 | 5 | 0/5 | 2.2 (0.51) | 101.2 (42.3) | 129.0 (52.8) |
| C | 18 | 9 | 6/6 | 14.04 (1.6) ^a | 1184.7 (225.6) ^a | 1344.2 (263.0) ^a |
| C | 0 | 9 | 0/6 | 2.46 (1.2) | 354.5 (109.3) | 378.2 (117.5) |
| D | 18 | 10 | 2/8 | 7.06 (1.49) ^a | 954.4 (162.7) ^a | 990.8 (187.5) ^a |
| D | 0 | 10 | 0/8 | 1.12 (0.27) | 213.6 (55.4) | 285.9 (70.7) |

C2 C12 subclones were implanted into irradiated and nonirradiated TA muscles of mdx nu/nu host mice. The radiation dose is given as grays (Gy). Values given for "tumor visible" are the number of legs that had macroscopically visible tumors out of the total number examined. The undifferentiated tissue is presented as a percentage of the area. The values in the last three columns are the mean with the SEM in parentheses.

^aStatistically significant difference (Mann-Whitney test) between values for the irradiated right TA and nonirradiated left TA.

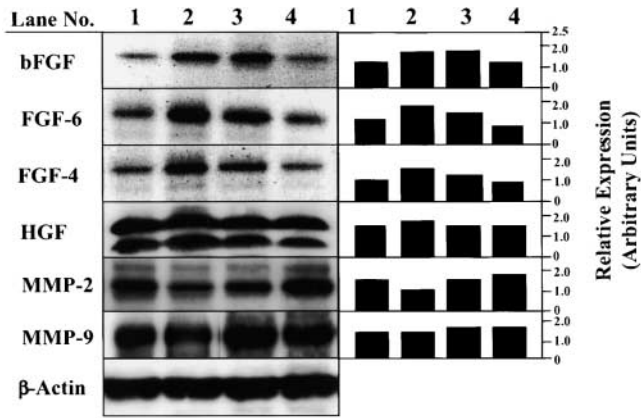


Figure 6. **Irradiation induces no significant changes in the expression of growth factors or MMPs in skeletal muscle.** Wild-type (C57Bl/10) and mdx skeletal muscle were irradiated (18Gy), and specific growth factor and MMP expression was evaluated by immunoblotting. Relative levels of expression for each protein were determined in comparison with β -actin as control (bottom). Lanes 1 and 3 are C57Bl/10 and mdx irradiated muscle, and lanes 2 and 4 are control C57Bl/10 and mdx nonirradiated muscle.

Discussion

Although radiation is often used therapeutically and as an experimental tool, its effect on living tissues is poorly understood. Here, we describe profound effects of a high dose of radiation on the interaction between irradiated recipient tissue and a population of nonirradiated cells subsequently grafted into that tissue. We find a significant augmentation of muscle formed from implanted conditionally immortal MPCs in both myopathic mdx nu/nu and normal C5⁻/ γ chain-deficient/Rag2⁻ mouse muscles that had been preirradiated with 18 Gy. A similar trend was found in the beige/nu/Xid mouse, but the difference was not statistically significant. Although the underlying mechanism responsible for this is not understood, in this mouse, it may be due to a lack of muscle response to the stimulus of irradiation.

The immortal C2 C12 myogenic cell line forms skeletal muscle in greater amounts than do conditionally immortal H2K18.30 MPCs, but also gives rise to tumors after several weeks in vivo (Wernig et al., 1991; Morgan et al., 1992; Pagel et al., 2000). Previous experiments have shown that expansion of engrafted C2 C12 cells in muscle is not constrained by normal myoblasts (Morgan et al., 1992), implying that the irradiation-induced proliferation is not simply a response to the creation of a vacant niche by ablation of endogenous satellite cells. Formation of C2 C12-derived neoplasms was considerably accelerated by preirradiation of the graft site. C2 C12 cells invariably gave rise to visible tumors within 3 wk in irradiated legs, but rarely in the nonirradiated, contralateral legs. This is reflected in the histology of the graft sites, which contained significantly larger undifferentiated interstitial cell areas in irradiated than in nonirradiated muscles in both mdx nu/nu and beige/nu/Xid hosts. The tumor-enhancing effect of radiation persists for at least 100 d and is dose dependent.

Our data imply that the effect of irradiation on enhancement of cell proliferation is restricted to the site of irradiation, for there is no systemic or contralateral effect, as has

been observed, for instance, in increased proliferation of proximal tubule cells in mouse kidneys contralateral to an irradiated kidney (Otsuka and Meistrich, 1993).

The mechanism by which irradiated tissue influences the proliferation of grafted cells is not clear. Ionizing radiation causes DNA damage, which, if not effectively repaired, causes cell death after the first or second postirradiation mitosis. Therefore, rapidly dividing cells, such as satellite cells in growing skeletal muscle, are more sensitive to irradiation than nondividing cells. Apart from preventing growth and regeneration of dystrophic muscle (Wakeford et al., 1991; Weller et al., 1991; Quinlan et al., 1995, 1997; Heslop et al., 2000), the effects of high doses of radiation delivered to mainly postmitotic skeletal muscle have scarcely been documented. Some increase in muscle fiber permeability in vitro has been reported (Canaday et al., 1994), but does not seem to occur in vivo (Pagel and Partridge, 1999). It has also been reported that the structural proteins titin and nebulin are degraded immediately after irradiation (Horowitz et al., 1986) and that microvascular networks within skeletal muscle are damaged by 10 Gy, causing a reduction of blood supply to the muscle 30 d later (Roth et al., 1999).

It is quite possible that radiation induces either muscle or nonmuscle cells within the graft site to produce growth factors that enhance donor cell proliferation. Indeed, radiation has been reported to raise levels of growth factors in various cells or tissues for up to 6 wk after irradiation (Peter et al., 1993; Yi et al., 1996; Gorski et al., 1999; Kruse et al., 1999; Mori et al., 2000; Wang et al., 2000). A survey of substances that may affect growth or migration of MPCs detected no elevation of β -FGF, FGF-4, FGF-6, MMP-2, MMP-9, or scatter factor (hepatocyte growth factor) in irradiated muscles. However, the crucial determinant may be the availability or efficiency of presentation, rather than the actual amount, of growth factor. This explanation is consonant with the long-lasting effect of irradiation, because a persistent change in the composition of proteoglycans in the interstitium of muscle might affect the presentation of growth factors by connective tissue elements and augment muscle cell proliferation (Desgranges et al., 1999; Stockholm et al., 1999).

Alternatively, radiation might ablate an inhibitory agent. Skeletal muscle is rarely the site of tumor formation (Hundt et al., 1999), and it contains a substance(s) that inhibits tumor proliferation (Bar-Yehuda et al., 1999). The notion of inhibitory control of cell proliferation is also in accordance with the fact that the muscle satellite cells are deeply quiescent in normal, mature, undamaged muscle, but are capable of rapid proliferation in response to injury. Moreover, simple removal from the muscle environment evokes extremely rapid activation of satellite cells (Beauchamp et al., 2000).

Effects of irradiated cells on the proliferation of nonirradiated cells in vivo have been noted previously. For example, nonirradiated mouse tumor cells mixed with irradiated cells and implanted subcutaneously into host mice grew more rapidly than the nonirradiated cells alone (Revesz, 1956, 1958). Similarly, tumor formation from mouse mammary epithelial cells was enhanced by irradiation (4 Gy) of the host mouse mammary glands before cell implantation (Barcellos-Hoff and Ravani, 2000), and survival, migration, and proliferation of implanted O2-A progenitor cells were en-

hanced by preirradiation (40 Gy) of the rat spinal cord graft site (Franklin et al., 1996). The proliferative effects of irradiation that we have shown here appear, from previous work on conditionally immortal myoblasts, to be restricted to a small subpopulation of cells that shows characteristics of early precursors (Beauchamp et al., 1999), perhaps corresponding to the “reserve” satellite cells described by Schultz (1996). It remains to be determined whether the C2 C12 cells that respond to the preirradiated graft site fall into the analogous nondifferentiating reserve cells that have been described in this line (Yoshida et al., 1998) and characterized by expression of CD34 (Beauchamp et al., 2000). In the context of current interest in circulating multipotential stem cells (Ferrari et al., 1998; Gussoni et al. 1999; Lagasse et al., 2000; Krause et al., 2001; Orlic et al., 2001), such a specific stimulatory effect may be important, because these cells have not been observed, so far, to make more than a rare and trivial contribution to myogenesis, even when directly injected into the muscle.

To our surprise, the switch to tumor formation in C2 C12 cells implanted into an irradiated environment was not irrevocable, because fragments of C2 C12–derived tumor produced very large amounts of muscle in a second host. Curiously, even in preirradiated sites in a second host, they produced more muscle than had been present in the original tumor, implying that they had been in some way altered by this first exposure to an irradiated environment. Thus, C2 C12 cells behave in an analogous way to primary (Yao and Kurachi, 1993) and conditionally immortal muscle cells (Morgan et al., 1994; Gross and Morgan, 1999) in that they function as MPCs after their implantation.

Our cloning experiments demonstrate that C2 C12 cells comprise a bimodal population in their propensity to form tumors *in vivo*. Initially, we implanted a mixture of retrovirally marked clones into irradiated mdx nu/nu mouse muscles and found a tumor in only one out of five muscles, examined 90 d later. This lack of tumorigenicity may have been due to the insertion of the retrovirus or the expression of β -gal, or again, exposure to geneticin rather than to the clonal selection itself. Immune rejection of cells marked with β -gal–expressing retroviruses (Abina et al., 1996; Visted et al., 2000) is unlikely in our immunodeficient mice, where we always find β -gal–expressing muscle fibers. Moreover, upon analysis of single clones, we found one retrovirally infected C2 C12 subclone that produced only muscle and gave rise neither to tumors nor to the excess of interstitial cells that are normally associated with grafts of C2 C12 cells. Other subclones gave rise to both muscle and tumor in irradiated legs. Of practical interest, the clone that gave rise to no undifferentiated tumor tissue provides an excellent model of muscle regeneration. The existence of nontumor-forming clones may explain the lack of tumors in previous experiments where C2 cells had been transfected *in vitro* and selected or cloned before their transplantation (Hamamori et al., 1994, 1995; Dhawan et al., 1996; Bohl and Heard, 1997). We have yet to determine whether the tumor-forming clone contains subclones that can still form muscle in a second host mouse.

The interaction between a cell and its environment in postnatal tissue is crucial to the fate of the cell. Environmen-

tal changes occur during differentiation, growth, and disease processes and after external damage to the tissue. We have shown that modulating the host environment by gamma irradiation before cell implantation augments muscle formation from MPCs by driving them into proliferation. Similarly, it drives proliferation of C2 C12 cells, but, additionally, it generates an accelerated neoplastic transformation that is reversible upon return of the cells to a nonirradiated environment. This demonstrates a profound phenotypic modulation by purely environmental factors. It may be akin to the changes from neoplastic behavior of teratoma cells and their normal integration into developing embryos (Mintz and Illmensee, 1975; Illmensee and Mintz, 1976) and the effect of the environment on the transformation of a normal cell into a malignant cell (for review see Park et al., 2000). Identification of the factor(s) within irradiated muscle that effects the proliferation of implanted cells would be important for the development of new strategies both for preventing tumor formation and improving myoblast transfer to treat myopathies such as Duchenne muscular dystrophy.

Materials and methods

Cell culture

The first experiments were designed to measure the amount of donor muscle formed in irradiated and nonirradiated host muscles. To standardize experimental conditions, we used a clonal muscle cell line for our donor cells, rather than primary MPCs, which show some variation between individual preparations. We used a male, conditionally immortal clone, H2K 18, derived from the *H2K^b ts A58* transgenic mouse (Jat et al., 1991) that proliferates at 33°C in the presence of gamma interferon and forms normal muscle (and not tumor) when implanted into mouse muscle (Morgan et al., 1994; Beauchamp et al., 1999; Gross and Morgan, 1999). Cells were grown as described previously (Morgan et al., 1994) and were infected with a retrovirus coding for cytoplasmic-localizing LacZ (Adam et al., 1991; Fassati et al., 1996) and recloned in geneticin (600 μ g/ml) to obtain a sub-clone that was myogenic, nontransformed, and 100% β -gal positive (H2K 18.30).

To assay the growth promoting effects of irradiation, we used C2 C12 donor cells (Yaffe and Saxel, 1977; Blau et al., 1983). C2 C12 cells were cultured in flasks, coated with 0.01% gelatin, in DME containing 10% FCS. In some experiments, C2 C12 cells were infected with the retrovirus coding for cytoplasmic LacZ and cloned in G418 to obtain clones that were 100% β -gal positive.

The above cell lines were kept as replicate vials frozen in liquid nitrogen. For each experiment, a single vial was thawed and cultured for 3 or 4 d before implantation. Pellets containing 5×10^5 cells were prepared for implantation.

Cell implantation

Dystrophin-deficient mdx mouse muscle undergoes extensive spontaneous degeneration and regeneration accompanied by infiltration of cells such as macrophages. To determine how the growth-promoting effect of irradiation is occurring, it is important to establish whether radiation has a similar effect on muscles that are not already undergoing these dynamic processes. We therefore compared two strains of nonmyopathic, immunodeficient mice to the mdx nu/nu host mouse as hosts for myoblast transplantation. The right legs of 3-wk-old mdx nu/nu (Partridge et al., 1989), beige/nu/Xid (Zietman et al., 1991), or C57 γ chain–deficient/Rag2^{-/-} (Goldman et al., 1998; Mazurier et al., 1999; Cooper et al., 2001) mice were irradiated with either 18, 9, or 4.5 Gy at a dose rate of 0.7 Gy/min (Gross et al., 1999). Control host mice had neither leg irradiated. 3, 28, or 100 d after irradiation, mice were anaesthetized either as before or with isoflurane, and cell pellets were injected with a Hamilton syringe, via a small skin incision, into the TA muscles of the left and right legs (Partridge et al., 1989).

For serial transplant of C2 C12–derived tumors, 5×10^5 C2 C12 cells were implanted into TA muscles of two mdx nu/nu hosts that had been preirradiated with 18 Gy. 3 wk later, the mice were killed and equal-sized pieces of tumor were excised from the TA muscles. These were implanted into a slit made in TA muscles of both legs of a fresh set of mdx nu/nu mice whose right legs had been irradiated with 18 Gy 3 d earlier.

Analysis of muscles

In mdx host mice, dystrophin may be used as a marker for muscle of donor origin. To enable us to identify muscle of donor origin in normal hosts, we marked the donor cells with a retrovirus expressing cytoplasmic-localizing LacZ. To validate this marker, we had to establish whether dystrophin and β -gal expression concurred in mdx nu/nu host muscles that had been injected with retrovirally marked donor cells.

Muscles injected with H2K 18.30 cells were removed for analysis 5 wk after cell implantation. Muscles injected with C2 C12 cells were removed for analysis 3 wk after cell implantation. This earlier time point was chosen for the latter experiment because tumors had formed in the irradiated legs by this time. Where myogenic cells had been injected into mdx hosts, the number of dystrophin-positive fibers in a representative cryostat section was counted (Morgan et al., 1993). Where donor cells were expressing LacZ, the number of fibers expressing LacZ was counted (Gross and Morgan, 1999).

The amount of tumor was calculated by estimating (from a random spot sample) the percentage of a representative cross section that was occupied by undifferentiated interstitial cells (Curtis, 1960).

To determine whether estimates of donor muscle achieved by counting the number of donor muscle fibers in a representative cross section tallied with measurements of the amount of donor DNA and β -gal activity present in a sample of a homogenate of the entire muscle, TA muscles from six female mice that had been injected with H2K 18.30 cells were removed and homogenized in 2 ml of DME. DNA was prepared from the entire muscle and the amount of male DNA was quantified by slot blotting (Beauchamp et al., 1999). In another experiment, TA muscles from seven female mice that had been injected with H2K 18.30 cells were removed, homogenized in DME, and an aliquot was taken for β -gal assay (Guerette et al., 1997). Mean values were compared by the Mann-Whitney test.

Growth factor and MMP expression in irradiated and nonirradiated muscle

A number of candidate factors involved in myoblast proliferation and/or migration were compared between irradiated and nonirradiated muscle by Western blot analysis. Both legs of 3-wk-old mdx and wild-type C57Bl/10 mice were irradiated with 18 Gy. Nonirradiated littermate mdx and C57Bl/10 mice were used as controls. 3 d after irradiation, muscles were removed, snap frozen in liquid nitrogen, and prepared for SDS-PAGE and Western blotting. In brief, muscles from four to five mice were pooled, homogenized in 650 μ l of 250 mM Tris HCl, 10 mM EDTA, pH 7.4, and fractionated (2–10 μ g of protein per lane) on 4–12% Tris-glycine SDS-PAGE gels (Invitrogen). Separated proteins were electroblotted onto Hybond C⁺ extra nitrocellulose membranes (Amersham Pharmacia Biotech), and the membranes were stained with Ponceau red (Sigma-Aldrich) before blocking by incubation in 5.0% nonfat milk in PBS. After blocking, antibodies (1–5 μ g per ml) against the following proteins were used to detect expression: hepatocyte growth factor (Cambio); β -FGF, FGF-4, and FGF-6 (R&D Systems); and MMP-2 and MMP-9 (Oncogene Research Products). Primary antibodies were detected using biotinylated species-specific secondary antibodies followed by the ABC system (Vector Laboratories). Protein components were visualized using the ECL system (Amersham Pharmacia Biotech) followed by exposure of the stained membrane to X-ray film (Eastman Kodak Co.). Protein levels were determined by scanning densitometry using the Grab-it software (UVP). Values presented are representative of three experiments and the expression levels are given relative to that of β -actin (Sigma-Aldrich) as loading control.

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