In Vitro Response of Hamster Melanoma KF Line to Combined Co-60 and Hyperthermic Treatments

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Abstract

In the interest of developing useful adjuncts to radiation therapy, the effect of Co-60 radiation combined with 42.0-42.5°C hyperthermia upon *in vitro* hamster melanoma cells was studied. The assay of cell survival following treatment was based upon the colony-forming ability of treated suspensions. The experiment was divided into two blocks. Treatments consisted of various doses of Co-60 radiation (0, 250, 500, 750, and 1000 rad), followed immediately by various durations of hyperthermic exposure (0, 15, and 30 minutes). Some literature reports indicate a possible synergistic effect between hyperthermia and ionizing radiation. The results of this study showed a mild hyperthermic effect upon *in vitro* cell survival, compared to the more dramatic effect of the Co-60 doses used. In one block only, significant reduction in cell survival was observed with 15 minutes of hyperthermia, but further reduction was not observed with a 30-minute duration. All levels of radiation dose significantly decreased cell survival. No significant interaction effect between the hyperthermia and ionizing radiation was observed, indicating the absence of any synergism with this treatment procedure.

Introduction

The administered dose in cancer radiotherapy is limited in part by the burden imposed upon healthy tissues adjacent to the tumor (3, 5, 9, 15). An adjunctive treatment which would increase the effectiveness of radiation while imposing a minimum of additional risk to healthy tissue would be beneficial and might even reduce the radiation dose required to control tumor growth. Elevated temperature, or hyperthermia, has been suggested as one mechanism for increasing the effectiveness of radiation therapy (9, 14). Hyperthermia alone has been found to have many effects. Nucleolus structural breakdown (10, 14) and varied effects on RNA synthesis (1, 14) have been reported at 45-46°C applied for 15-60 minutes. Hyperthermic killing of cells cultured *in vitro* over several generations exhibits a large temperature dependence (12, 13). Significant reduction in surviving cell numbers is not often seen at temperatures below 41°C. The effects of various radiation and hyperthermia combination treatments have been studied using many different mammalian lines, both normal and neoplastic, cultured *in vitro* (4, 6). There has been some indication that the inclusion of the hyperthermia may produce a synergistic effect with the ionizing radiation.

The objective of this study was to determine the effect of ^{60}Co radiation combined with 42.0-42.5°C hyperthermia upon cell viability of KF hamster melanoma (7) *in vitro*. Cell viability was measured by the ability of treated and control cultures to form colonies in a modified Puck-Marcus titration (13). Statistical analysis of these surviving fractions was used to differentiate significant differences in treatment effects.

Materials and Methods

Design

The experiment was conducted in two blocks or runs. In each run, 45 vials of cells were treated. Five levels of ^{60}Co dose (0, 250, 500, 750, and 1000 rad) and three durations of 42.0-42.5°C hyperthermia (0, 15, and 30 minutes) were used in a completely randomized block design with three vials in each treatment combination per run. Radiation treatment preceded hyperthermia. After the conclusion of treatments, the sample suspensions of cells were diluted and plated in culture dishes. After 10 days of incubation, the cells in these platings capable of replication had produced colonies which, upon staining, were readily distinguishable under a 1-2 X magnification. An estimate of the viable cell concentration of the post-treatment suspensions was obtained by dividing the counts of colonies per plate by the dilution plated.

Cell Cultures

Cell cultures were maintained in Eagles Minimum Essential Medium supplemented with 10% fetal calf serum and antibiotics. Cultures were incubated at 37-38°C with a CO_2 enriched atmosphere. About 60 ml of a suspension containing 1.1 million cells per milliliter was prepared for each run. To each of 45 glass 1-dram vials per run was added 0.75 ml of the suspension. These vials were randomly assigned to treatments. Treated and control suspensions were plated in conditioned medium in 60-mm diameter plastic tissue culture dishes. Conditioned medium (medium drawn off healthy cultures, sterilized by filtration, and mixed with an equal part of fresh, sterile medium) enhanced the colony formation ability of healthy isolated cells in preliminary trials. The 60-mm culture dishes enabled up to 300 colonies to be distinguished. The minimum number of colonies per plate was maintained at 100 by careful dilution to minimize statistical error in counting. All vials were returned to the incubator following hyperthermic treatment until they were diluted and plated in the groups of three vials per treatment combination.

Treatments

The ^{60}Co radiation dose rate was 32 rad per minute. Since it was possible that the length of time between the radiation and hyperthermic treatments was crucial, the irradiation was concluded simultaneously for all vials in each run, and heat treatment was begun simultaneously. Thus, all vials which were to receive a 1000-rad total dose were spaced around the 50-cm radius circle and given a 250-rad dose. The vials to receive 750 rad were added to the circle and another 250-rad dose was delivered. All total doses were similarly composed of 250-rad increments. This short fractionation of the dose did not result in a detectable change in the viable cell concentrations. All vials, including the ^{60}Co controls, were kept at room temperature throughout the radiation exposure period.

Hyperthermia was administered at 42.0-42.5°C in a well-circulated water bath. Five mercury thermometers with an accuracy of about ±0.2°C were used in different locations in the bath. Vials were simul-

taneously submerged approximately halfway in plastic holders. A thermometer in a similar but uncapped vial was used to monitor cell culture temperature inside the vials. Mechanical timers were started when the temperature of the cell cultures reached 41.5°C.

After 9-10 days of undisturbed incubation, the plates were dehydrated for 10 minutes in methanol and stained for 10 minutes in Giemsa stain. The definition of a colony to be counted was based upon the number of healthy cells associated as a group. All groups of 15 or more cells were counted. Although the number in association was often much more than 15, these groups were counted as one colony unless two or more cores indicative of separate progenies were evident.

Results and Discussion

Two dilutions of each vial were plated. As a screening procedure, each plate was counted, and the dilution with between 100 and 300 colonies was chosen to represent each treatment group of three vials. A total of 90 plates was thus selected representing the 45 vials from each of the two runs. These were recounted in a random and blind manner. The observed counts of colonies per plate were divided by the dilution factor to determine the viable cells per milliliter for each vial. Means and standard deviations are shown in Table 1.

TABLE 1. *Means and standard deviations of survival data.*

Duration of hyperthermia (minutes)	Radiation dose (rads)				
	0	250	500	750	1000
		Block 1			
0	115,000[a]	47,900	19,200	7,730	3,250
	±5,570	±15,100	±6,000	±1,040	±190
15	136,000	90,400	18,600	12,300	2,990
	±27,500	±11,600	±1,310	±4,700	±1,020
30	125,000	96,700	11,100	3,290	1,050
	±6,080	±9,880	±1,750	±980	±390
		Block 2			
0	178,000	46,000	20,300	7,750	4,790
	±28,400	±9,320	±1,920	±2,330	±1,210
15	48,800	41,600	8,500	3,980	2,710
	±10,300	±13,900	±2,090	±610	±150
30	62,800	41,400	12,900	4,390	1,630
	±4,660	±6,130	±1,210	±1,400	±370

[a]Data are expressed as viable cells per milliliter.

The sample variance depended upon the dilution factors which ranged over three orders of magnitude. This dependency resulted in a lack of homogeneity of variance. Use of the log transformation alleviated this problem, as indicated by the Burr-Foster Q test (2). The analysis of variance (2) for these data was performed with the Purdue University Computer Center Statistical Package for the Social Sciences. The results of this analysis are presented in Table 2. Two F values

are shown, F_1 generated by dividing all other mean squares by the error mean square and F_2 generated by dividing the mean squares for the main effects and two-way interactions by the mean square from the three-way interaction.

TABLE 2. *F-Tests for significance of main effects and interactions*[a].

Source	d.f.	MS	F_1	F_2
B	1			
H	2	0.349	33.3*	8.73*
R	4	8.149	777 *	204 *
BR	4	0.083	7.87*	2.08
BH	2	0.312	29.7*	7.80
HR	8	0.103	9.85*	2.58
BRH	8	0.040	3.84*	----
E	60	0.010	----	----

B = main effect due to the block design.
H = main effect due to the heat treatment duration.
R = main effect due to the radiation dose.
BR = interaction effect due to block-radiation dose.
BH = interaction effect due to block-heat treatment duration.
HR = interaction effect due to radiation dose-heat treatment duration.
BRH = three-way interaction effect due to block-radiation dose-heat treatment duration.
E = estimate of random error.

[a]F_1 values are based on use of MS(E) in denominator. F_2 values are based on use of MS(BRH) in denominator. Significance of the F statistics is based on a 0.99 confidence level ($\alpha = .01$) and is indicated by an asterisk.

The comparatively small value of the error mean square suggests the possible underestimation of the random component. The three-way interaction term should be less susceptible to any bias influencing the random error estimation, and is at least as large as the random error. Therefore, the analysis utilizing the three-way interaction mean square as the error estimate, rather than the observed error mean square, is the more confident choice. Use of F statistic calculations at a 0.99 level of confidence showed both main effects of radiation dose and heat duration to be significant, but none of the interactions were significant. However, the interaction between heat duration and the block effect was significant at a 0.95 level of confidence. This interaction prevented pooling of data across the two runs, and Newman-Keuls range tests to determine which levels of the factors were significantly different were performed separately for the two runs. The results of these tests are presented in Table 3.

In only one run did the range tests show a significant reduction in cell viability following the 15-minute hyperthermia duration, and in this run the 30-minute duration had no further effect. The temperature used is believed to be near the minimum required for an effect with these durations (6, 11, 12). The desire to present a minimal insult to healthy tissues prompted the choice of a mild temperature. It is quite likely that temperatures in excess of 42.5°C would produce a more dramatic effect on cell survival. All levels of ^{60}Co radiation dose used

resulted in significantly different cell survival. Since the heat duration-radiation dose interaction was not significant, no evidence of a synergism between the treatments was demonstrated by these procedures.

Although many investigators (4, 9) have reported evidence of significant synergism between hyperthermia and radiation exposure, many others (6) have, as does this study, reported data to the contrary. Experimental procedures and dose levels are likely to affect the significance of synergism. Further work is needed to help elucidate how and when heat treatment may be beneficially used in cancer therapy programs. Further *in vitro* studies can aid in ascertaining the temperature, radiation dose, treatment protocol, and other factors necessary for the efficient use of hyperthermia in cancer therapy.

TABLE 3. *Newman-Keuls ranking of survival means for MS(BRH) analysis*[a].

	Levels of radiation dose (rads)				
	0	250	500	750	1000
Run 1	125,000[b]	78,300	16,300	7,760	2,430
Run 2	96,400	43,000	13,900	5,370	3,040

	Levels of heat duration (minutes)		
	0	15	30
Run 1	38,600[c]	52,000	47,000
Run 2	51,300	21,100	24,600

[a] Underscoring indicates a nonsignificant difference at the 95% confidence level.
[b] Mean viable cells/ml for 9 samples.
[c] Mean viable cells/ml for 15 samples.

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