In vivo dose response assays

Tumor assays

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1. **Tumor growth measurements; tumor growth delay.**

   - After irradiation, the tumor is measured daily to determine the mean diameter, or volume.

   - Plot tumor size versus time after treatment.

   - Unirradiated tumors will grow continuously.

   - Irradiated tumors will show some shrinkage or delayed growth, then regrow.

   - Score *growth delay* or *time to grow to a specified size*; plot versus dose.

   - Dose Modification Factor: use of growth or growth delay curves to measure the effect of an added agent or treatment e.g., a **radiosensitizer**.

2. Tumor Control (TCD$_{50}$ assay)

- Irradiate tumors of uniform sizes with various doses. Observe for local control or recurrence. Plot % control vs. dose.

- TCD$_{50}$ = dose to control 50% of tumors

- This is a more relevant assay for radiotherapy than growth delay, but requires keeping greater numbers of animals for longer periods of time so is more costly.

1, 2 or 10 doses: 24 hours between fractions, shift in TCD$_{50}$ indicates an extensive repair of sublethal damage.

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3. Dilution assay technique

- Inject a known number of leukemia cells from a donor mouse into a recipient mouse to determine the number of cells needed to cause leukemia.
- TD_{50} = number of cells needed to cause leukemia in 50% of recipient animals.
- Irradiate donor mouse, collect and count leukemic cells, inject i.p. into recipient mouse, determine TD_{50}.
- Surviving fraction = (Control TD_{50})(Irradiated TD_{50})

Dose response *in vivo*: dilution assay technique for various murine tumors plus/minus oxygen.

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Dilution assay:
- Various numbers of tumor cells from donor animal injected into groups of recipient animals.
- A determination of the number of cells required to induce tumors in 50% of the recipients is made (TD_{50}).
- Control TD_{50}/test TD_{50} = SF

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4. Lung colony assay

- Same idea as the dilution assay, except uses solid tumor cells rather than leukemia cells.
- The tumor is irradiated in a donor animal, removed and single cell suspension prepared, cells injected into recipient animal.
- About 20 days later, lung colonies are counted.

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Lung colony assay system:
- Tumor irradiated *in situ*, then excised and made into single cell suspension.
- Known numbers of cells injected i.v.
- The number of lung colonies is a measure of the number of clonogenic tumor cells in the injected suspension.
- Compare to control; plot survival curve
- Mist inject same total number of cells: mix cells with large number of "carrier" heavily irradiated cells.

5. *In Vivo-In Vitro* assay
- Some tumor cell lines have been adapted to grow both *in vivo* and *in vitro*.
- Irradiate tumors in animals, remove tumors, prepare single-cell suspension, plate cells in suitable medium for colony formation.
- There is not necessarily qualitative or quantitative agreement between results of this assay and results obtained when tumors are left *in situ*. Possible reasons include PLDR or differences in types of damages expressed.

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Xenografts- tumor growing in a different species host. Must suppress host immume system:
- nude mice: lack thymus
- scid mice: lack B and T cells
- radiation or drugs can suppress immune system

Advantages
- Cells retain the human karyotype and some dose-response characteristics of the tumor from which they were derived.
- For many tumors growth delay and clinical remission rate correlate.

Disadvantages
- Possible rejection by host, may cause misleading results, especially if the endpoint is tumor control.
- Cells may change; kinetic changes, selection.
- Host is different, absence of an immune response is artificial.
- Stromal tissue different, rodent origin, makes studies where vascularity is important questionable.

6. **Spheroids**

An *in vitro* tumor model system

Certain cell types growing in suspension or in soft agar will aggregate and remain in contact, forming multicellular, 3-D spheres called spheroids.

Spheroids rely on diffusion of O_2 and nutrients from the media. As they grow larger, they will develop nutrient-deprived centers.

Useful as a model of micrometastatic tumors

Larger spheroids generally contain 3 types of cell populations
- Asynchronous, cycling cells
- Noncycling cells
- Noncycling, hypoxic cells

Growth methods
- Spinner flasks
- Liquid overlay

Advantages

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- Diffusion (O_2 , small molecules, radiolabelled agents, MAb)
- nutrient deprived centers
- hypoxia in center
- model for tumors
- can be irradiated
- growth can be monitored
- disaggregate for analysis of cell survival
- cells in spheroid more radioresistant than same cells *in vitro*, small spheroids may have the same D_0 as single cells.

Normal Tissues: Dose response assays

Three basic types
- direct clonogenic assay
- functional assay
- multifraction experiments used to assemble dose-response relationship or α/β ratios

Direct clonogenic assays

Skin Clones Assay
- Thirty-plus years ago, skin damage was a major concern in radiation therapy because of the use of orthovoltage X-ray machine. Now, skin damage is much less of a clinical issue due to the use of linacs and MeV energy photons.
- Technique: Pluck hair from mouse's back, cover center of plucked area with a lead disk, irradiated surrounding area with high dose (e.g. 30 Gy) to create a moat of dead cells.
- Remove lead disk and irradiate central test area with varying doses.
- After time has been allowed for the skin to regrow, count patches, or islands, of skin regrowth.
- Each patch represents a clone formed from a single surviving stem cell.
- Construct a survival curve by plotting number of surviving clones vs. dose.

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N.B. Actual number of cells in skin region is not known, only the number of colonies. Width of shoulder can be estimated with split dose experiment. Separation of the two lines is D_q.

Crypt cell assay

- Crypt cells constantly dividing
- These are the renewing stem cells in the intestinal epithelium

Procedure
- Deliver a total body dose of 11-16 Gy, which kills most crypt cells but spares the cells in the villi.
- As villi cells are lost by normal migration and sloughing off processes, no functional cells replace them: the villi shrink.
- At 3-4 days post irradiation the crypts begin to regenerate.
- Animals sacrificed, cross sections of intestines stained and scored.
- Plot ***number of regenerating crypts per circumference*** *vs* dose.

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Similar assays can be used for testes stem cells and kidney tubules.

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Crypt cell survival curves, single dose and multiple doses
- D_0 for 1 fraction = 1.3 Gy.
- Shoulder is large: D_q = 4-4.5 Gy (large capacity for repair).

Limitations:
- Does not directly measure surviving fraction.
- Useful dose range 11-16 Gy: need enough kill to measure individual regenerating crypts.

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Cells transplanted to another site

Spleen colony assay
- Similar to dilution assay except uses **_normal_** bone marrow cells, rather than leukemia, and colonies form in the spleen.
- To conduct experiments, irradiate donor mouse to different doses, remove bone marrow cells, count, and inject known number of cells into supra-lethally irradiated mice (spleens sterilized).
- 9-10 days later remove spleen and count nodules.
- Calculate surviving fraction as SF = (# colonies counted)/(# cells inoculated x "PE"). Plot SF versus dose for survival curve.

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Recipient mice treated with 9 Gy, supralethal dose sterilizes spleen cells.

~ 100 cells injected to form 1 spleen colony.

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Note that the normal bone marrow cells are relatively radiation sensitive, with both a small D_0 and small n.

Spleen colony assay for bone marrow cells

Fat pad assay
Normal thyroid gland cells

Also used for mammary gland

24 hour delay before removing thyroid gland reveals repair of potentially lethal damage (PLD).

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Summary of normal tissue sensitivity

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Human A-T cells are a DNA repair-deficient mutant: one of the most radiation-sensitive human cell lines.

BM-CFU, mammalian thyroid cells: transplant into another tissue in recipient animal for assay.

Shoulder width is the principal variable.

Normal Tissues: Functional assays

Not a direct measure of cell survival, but ***direct relevance to clinical side effects.***

Skin reaction

Pig Skin
Very similar to human skin: Color, hair follicles, sweat glands subcutaneous fat.

- Set up an arbitrary scale for scoring skin reactions.
- Irradiate skin regions with various doses.
- Score skin reaction as a function of time.

TABLE 18.1. *Radiation Reactions in Pig Skin*

Arbitrary Score	Reaction
0	No visible reaction
1	Faint erythema
2	Erythema
3	Marked erythema
4	Moist desquamation of less than half the irradiated area
5	Moist desquamation of more than half the irradiated area

From Fowler JR, Morgan RL, Silvester JA, Bewley DK, Turner BA: Experiments with fractionated x-ray treatment of the skin of pigs: 1. Fractionation up to 28 days. Br J Radiol 36:188–196, 1963, with permission.

Two waves of skin reaction

Early: 10-40 days

Late: gradual increase to a broad maximum 50-100 days, more severe damage.

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Dose response is obtained by plotting skin reaction *vs* dose.

Fractionation results in considerable sparing.

Other Functional Endpoints

A variety of other functional assays have been used:
Breathing rate, spinal cord myelopathy, bladder function, tear production, etc.

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Note the dramatic sparing from fractionation

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Lethality (LD$_{50}$ assay)

- LD$_{50}$ (mean lethal dose) = dose required to kill 50% of the animals in a given time period, e.g., 30 days, LD$_{50/30}$
- Usually implies total body irradiation.
- Can also be determined after irradiation of a portion of the body.

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Mortality of rhesus monkeys following single total-body exposure to x rays.

Death is from bone marrow depletion.

Multifraction Experiments

Early responding tissues: skin, intestinal epithelium, bone marrow
These tissues are rapidly dividing, self-renewing systems.

Late responding tissues: spinal cord, lung, kidney

"Radiation response of all tissues results from depletion of critical parenchymal cells." Hall, 2000

Early/late differences are a function of the critical cell turnover rate.

Unanswered question: role of vascular damage *vs* parenchymal cell depletion.

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This approach allows estimation of α/β ratios in tissues with non-clonogenic endpoints.