

Cell Survival Curves

Cell death

A cell that is able to proliferate indefinitely and form a large colony from a single cell is said to be **clonogenic**.

Tumor cells can be grown indefinitely in cell culture.

Normal cells must be **transformed** to grow indefinitely in culture.

For cells growing in culture, the loss of the ability to continue growth is termed **reproductive death**.

Following irradiation, cells may still be intact and able to produce proteins, synthesize new DNA and even go through one or two cell divisions. But if it has **lost the capability to reproduce indefinitely**, it is considered dead.

Very high radiation doses (10,000 rads or 100 Gy) can cause the breakdown of all cellular functions.

In contrast, the **mean lethal dose** for loss of reproductive capability is usually less than 2 Gy.

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Clonogenic Survival Assay:

- Cells from an actively growing stock are harvested by gentle scraping or by the use of trypsin.
- The number of cells per unit volume is determined manually (hemocytometer) or electronically (Coulter Counter).
- Known numbers of cells can then be plated into fresh dishes. If allowed to incubate for 1-2 weeks, clonogenic cells will form macroscopically visible colonies that can be fixed, stained and counted.
- Not every cell seeded will form a colony, even in the absence of irradiation, due to factors such as errors in counting, stress of manipulation, suboptimal growth medium, etc. The **plating efficiency (PE)** is defined as the number of colonies observed/ the number of cells plated.

- $$PE = \frac{\textit{colonies observed}}{\textit{number of cells plated}}$$

- Parallel dishes are seeded with cells that have been exposed to increasing doses of radiation. The number of cells plated is increased so that a countable number of colonies results. **Surviving fraction (SF)** is the colonies counted divided by the number of colonies plated with a correction for the plating efficiency.

- $$SF = \frac{\textit{colonies counted}}{\textit{cells seeded} \times (PE / 100)}$$

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Cell survival curves

Cell survival data are generally plotted as logarithm of the surviving fraction versus dose.

For comparing curves, it is convenient to represent them mathematically, based on hypothetical models for the mechanisms behind lethality.

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The interpretation of the shape of the cell survival curve is still debated, as is the best way to fit these types of data mathematically.

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Radiation sensitivity and the cell cycle

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Example of cell cycle times

Cell cycle phase	CHO hamster	HeLa human
T_C	11	24
T_M	1	1
T_S	6	8
T_{G1}	3	4
T_{G2}	1	11

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Target Theory

Target theory originated from work with *exponential dose response curves*.

It was assumed that each “hit” results in an inactivation, i.e., a “single-hit, single-target model”.

- Each cell has a single target.
- Inactivation of the target kills the cell.

Linear Survival Curves

Irradiation of cells with high-LET radiation produces linear survival curves. The relationship between the surviving fraction S and the dose D is then:

$$S = e^{-\alpha D} \quad \text{where:}$$

S is the number of surviving cells,
 $-\alpha$ is the slope, and
 D is the radiation dose delivered.

This relationship is more commonly represented as $S = e^{-D/D_0}$
by defining D_0 as $1/\alpha$.

When $D = D_0$, $S = e^{-1} = 0.37$

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Poisson Distribution

All calculations of hit probability are governed by Poisson statistics, where the probability of n events is given by

$$P(n) = \frac{(e^{-x})(x^n)}{n!}$$

where x = the average number of events
and n = the specific number of events

If each “hit” is assumed to result in cell inactivation, then the probability of survival is the probability of not being hit, $P(0)$.

From the Poisson relationship, where $x = 1$, and $n = 0$,

$$P(0) = \frac{(e^{-1} \cdot 1^0)}{0!} = e^{-1} = 37\%$$

For this reason, D_0 is often called the *mean lethal dose*, or the dose that delivers, on average, *one lethal event per target*.

Exponential dose response relationships are found in certain situations

- Certain types of sensitive cells (e.g., haemopoietic stem cells)
- Synchronized populations in M and G₂
- Irradiation with high-LET radiation

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Cell Survival Curves with Shoulders

Survival curves for most mammalian cells exposed to **low-LET radiation** show some **curvature**.

The initial low dose region in which there is less cell inactivation per unit dose than at high doses is called the **shoulder**.

Often the higher-dose region tends towards a straight line.

The parameter **D_0** can then be used to characterize the radiosensitivity in the linear (high dose) region of the curve.

Extrapolation of the terminal straight line portion of the curve back to the abscissa defines a value, **n** , the **extrapolation number**.

In the shoulder region of the curve the proportion of the cells killed by a given dose increases with the dose already given. Two interpretations are possible:

- Cell death results from the **accumulation of events** that are individually incapable of killing the cell, but which become lethal when added together (target models).
- Lesions are individually repairable but become irreparable and kill the cell if the **efficiency of the enzymatic repair** mechanisms diminishes with number of lesions and therefore the dose (repair models).

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Linear-Quadratic Model (two component model):

The linear quadratic model has evolved from two similar formulations, each with roots in target theory.

Theory of Dual Radiation Action

- Lesions responsible for cell inactivation result from the interaction of sublesions.
- At least two sublesions are required for cell inactivation.
- Sublesions can be produced by the passage of one or two radiation tracks.

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Molecular Theory of Cell Inactivation (Chadwick and Leenhouts, 1981)

- Cell inactivation results from unrepaired DNA double-strand breaks.
- At low-LET, a dsb can result from either a single event (linear component) or two separate events (quadratic component).
- Alternatively, cell inactivation results from chromosome aberrations.
- Some aberrations are produced by a single event.
- Some aberrations are produced by two separate breaks.

Observations of chromosome damage led to the assumption that since DNA has 2 strands, it must take two events to break a strand.

Linear-quadratic model

The linear quadratic model assumes that a cell can be killed in two ways.

- Single lethal event
- Accumulation of sublethal events

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If these modes of cell death are assumed to be independent,

$$S = S_1 S_2$$

Where S_1 is the **single event**

killing or $e^{-\alpha D}$

And S_2 is the **two event killing** which can be represented as $e^{-\beta D^2}$

The most common expression is

$$S = e^{-(\alpha D + \beta D^2)}$$

S is the fraction of cells surviving a dose D and α and β are constants.

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Linear-quadratic model

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Useful parameters from linear quadratic cell survival curves:

- D_1 , the **initial slope**, due to single event killing, the dose to reduce survival to 37%
- D_0 , the **final slope**, interpreted as multiple-event killing, the dose to reduce survival by 67% from any point on the linear portion of the curve.
- some quantity to describe **the width of the shoulder**. The extrapolation of the final slope D_0 , back to the y axis yields n , the **extrapolation number**. The larger the value of n , the larger the shoulder on the survival curve.

Other Target Models

Single-hit, multi-target model:

Assumptions:

- Each cell contains n distinct and identical targets.
- Each target can be inactivated by the passage of a charged particle (a hit).
- Inactivation of a target is a **sublethal** event.
- All n targets must be inactivated to kill the cell.
- For a dose D_0 there is on average **one hit per target**.

Therefore, for a dose D_0 , the probability that a target is undamaged is e^{-D/D_0}

If the probability that a target survives is $S = e^{-D/D_0}$, then the probability that the target is hit is:

$$P(h) = 1 - e^{-D/D_0}$$

and the probability that all n targets are hit is

$$P(h) = (1 - e^{-D/D_0})^n$$

Therefore the probability that all targets will not be hit, i.e., the probability of survival, is:

$$S = 1 - (1 - e^{-D/D_0})^n$$

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[Alpen, 1990]

As $D \rightarrow \infty$, $S \rightarrow ne^{-D/D_0}$, the linear portion of this curve thus yields a slope of $-1/D_0$, and a y intercept of n (extrapolation number).

Thus, in this target theory model, the parameters D_0 and n define the radiosensitivity of the sublethal targets and their number (n) per cell.

Quadratic model (two hits, single target):

Single target

Two independent “hits” are required for inactivation.

The mean number of lethal events is then proportional to the square of the dose.

$S = e^{-\beta D^2}$, where β is the parameter relating dose to the probability of a lethal event.

These two models (single hit, multi target; two hits, single target) are good representations at high doses.

But they predict almost zero slope at low doses (underestimate the effect of low doses).

Models based on Repair

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Linear survival curves are easy to understand

The curvature in the shoulder continues to be “interpreted”

Repair is definitely involved...

Classic split-dose experiments, Elkind and Sutton, 1959

Two doses of low-LET radiation

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Low-LET followed by high-LET

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High-LET followed by low-LET

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Other Dose Response Assays

Mitotic arrest: cell division delay

- Block of progression of cells through the cell cycle
- Cells are usually blocked in the G2 phase of the cell cycle (G1 also possible)
- The block is usually temporary, length of block depends on radiation dose.

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Apoptosis: programmed cell death

A distinct mode of cell death, different from necrosis in terms of morphology, biochemistry and incidence. Generally considered to be an active process of gene-directed self-destruction.

Normal process for removal of cells.

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Occurs spontaneously in both normal tissues and tumors.

Endpoints used to measure apoptosis: microscopy, TUNEL, flow cytometry, specific staining (caspase, annexin)

Multiple pathways are involved in apoptosis.

Different pathways may be involved in the same cell type depending on the stimulus as well as in the response of different cell types to the same stimulus.

Interruption of normal apoptosis pathway is involved in many cancers.

Comparison of Features of Apoptosis and Necrosis

<u>Feature</u>	<u>Apoptosis</u>	<u>Necrosis</u>
Morphology	condensation of cell	swelling, lysis
Membrane integrity	persists until late	early failure
Mitochondria	unaffected	swelling, Ca uptake
Other organelles	remain intact	swelling
Chromatin	condensed, electron dense	granular
Appearances	affects scattered individual cells	usually affects tracts of contiguous cells
Inflammation	usually absent	usually present

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Growth rate assays to measure cell survival

Examples: direct cell counts
Incorporation of radiolabeled biochemical substrates (e.g., ^3H -Thd)

Tetrazolium (MTT) colorimetric assay

Quantification of cell growth based on assumption that only viable cells reduce MTT to a blue formazan product. Amount of product (color) is directly proportional to number of viable cells.

Procedure:

Cells plated in multiwell plates, treated with radiation (or drugs), allowed to grow for a period of time. Cells are then treated with MTT, solubilized and absorbance is measured.

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Some Advantages:

- Relatively simple and rapid
- Requires small number of cells
- May be semiautomated

Some Disadvantages:

- Direct correlation to clonogenic assays requires great attention to experimental details.
- Technical difficulties doing experiments in hypoxia
- Only accurate over a 1-1.5 log of response
- Cannot distinguish normal from tumor cells in a biopsy.

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Current area of active research: low dose hypersensitivity/adaptive response

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Effects of Radiation on Chromosomes

Chromosome damage can be:

- morphologically visible, e.g., changes in number or structure
- or not visible but with functional consequences: mutations.

Methods of chromosome analysis:

- Standard staining after adding an agent that blocks mitosis in metaphase.

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- Banding techniques: various techniques to stain the chromosomes produce visible bands.
- Incorporation of [^3H]Thd followed by autoradiography.

Techniques to look at chromosome aberrations

Premature chromosome condensation:

- Irradiate cells
- Fuse irradiated cells with mitotic cells
- Factors in the mitotic cells **force** the chromosomes of the irradiated interphase cells to condense.
- This visualizes all damage including some that may have been repaired.
- Visualizes severe damage that may have been detected before mitosis and prevented the cell from entering mitosis.

Micronucleus formation

- Chemically block cytokinesis to yield binucleated cells.
- Stain DNA.
- Fragments of chromosomes with no centromere not associated with nuclei are micronuclei.
- Score % of binucleated cells with micronuclei.

Fluorescence in situ hybridization (FISH):

Uses fluorescently tagged chromosome probes (complimentary DNA) to specific chromosomes or regions of chromosomes.

Types of chromosome damage observed

- Terminal deletions
- Intrachromosome exchange
- Interchromosome exchange

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Chromosome aberrations and cell death

- Chromosome aberrations can be detected after doses as low as 0.1 Gy.
- Chromosome aberrations reflect both the initial damage and the repair. (misrepair), because the chromosomes are not visible until the cells enter mitosis.
- Doses in the 0.5 – 2 Gy range, produce on the average one chromosome aberration per cell.
- This dose range is, on the average, the mean lethal dose for cells.
- The frequency of chromosome aberrations is a linear quadratic function of radiation dose.
- There are considerable data showing a relationship between cell killing and the induction of chromosome aberrations.

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Historically, these led to the theory of dual radiation action.

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