Modulation of receptor coupled signalling pathways – A novel approach in cellular radioprotection –

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1 Introduction

Professor Dr. Hanns Langendorff (1901–1974) accepted his "Ruf als Direktor des Strahlenbiologischen Instituts an die Universität Freiburg" in 1936, the year that I was born. He was the first Radiobiology Professor and held this chair until 1971, the year that I entered the field of Radiobiology at the Department of Radiopathology at the University of Groningen! Coincidence?

In a certain way I followed his footsteps and even worked at his laboratory in Freiburg (Director Prof. Streffer) the year after he died. So you may understand that I'm proud and thankful to receive the medal in his honour.

As a subject for this lecture I choose a recent development in the field of cellular radioprotection that can be considered as a follow up of our earlier work on the radiosensitivity of biomembranes and its meaning for cell functioning.

2 Membrane damage versus DNA-damage as cause for cell death

Although DNA has always been considered as a main target responsible for radiation induced cell killing, in the late sixties and early seventies there was a strong suspicion that some non-DNA structures might also play a role under certain conditions and in certain cellular systems. The most popular candidate proposed for this role has been the membranes structure of the cell. Alper [1] stressed the possibility that membrane lipids might be the "O" type target and DNA the "N" type target. She postulated that a high OER corresponds with substantial "O" type (membrane) damage in the cell, while a low OER relates to relatively more "N" type (DNA) damage. It was a creative idea, worth pursuing in order to obtain evidence for or against it. A review of this pursue has been published in 1987 [10].

We now know that membrane damage only contributes significantly to the mode of cell killing when cells do not divide or do proliferate very slowly and also when dividing cells are irradiated with very high doses (> 20 Gy single dose). A high OER as such is no indication for the importance of non-DNA structures as critical targets. Lipid peroxidation is however oxygen dependent. When these reactions are determining cell damage leading to cell death, very high OER's can be found [5, 8] which are dependent on dose rate.

3 Lipid peroxidation, membrane damage and dose rate

When DNA damage determines cell death low dose rates allow repair during (low LET) radiation. So less cells will die at a certain dose when a low dose rate is applied.

For the process of lipid peroxidation the opposite is the case. More ultimate damage will result from low dose rates. This was discovered by us [5] in the late seventies after irradiation of liposomes. An example of the results of such an experiment is given in figure 1. Specifics of this and related experiments can be found in the literature [8–12].

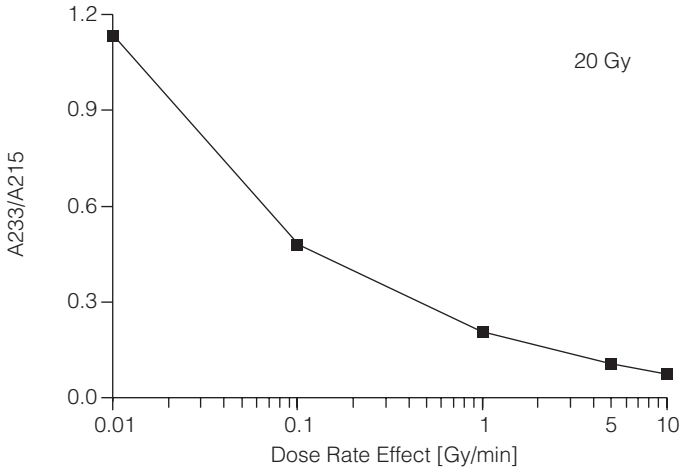

Fig. 1: X-irradiation of liposomes. MLV´s were prepared and lipid peroxidation measured as published [5, 8].

The inverse dose rate effect in lipid containing membranes can be explained by the existence of chain reactions in the phospholipid bilayer. The scheme of radiation induced lipid peroxidation consists of initiation, propagation and termination reactions.

Initiation: $RH + OH\cdot \rightarrow R\cdot + H_2O$

Propagation:
$R\cdot + O_2 \rightarrow RO_2\cdot$
$RO_2\cdot + *RH \rightarrow RO_2H + *R\cdot$
$*R\cdot + O_2 \rightarrow$ etc. (chain reaction)

Termination:
$RO_2\cdot + *RO_2\cdot \rightarrow (RO_2)_2$
$R\cdot + *R\cdot \rightarrow R_2$
$*R\cdot + RO_2\cdot \rightarrow *RO_2R$

RH represents an unsaturated fatty acid or acyl chain, H being a labile methylenic hydrogen. The alkyl radical (R·) reacts with oxygen and forms a peroxy radical ($RO_2\cdot$). When $RO_2\cdot$ is in the proximity of another unsaturated fatty acid or acyl chain (*RH), the peroxy radical ($RO_2\cdot$) will abstract a methylenic hydrogen atom from *RH, thus yielding a

hydroperoxide (RO_2H) and a new alkyl radical *R·, which may react with oxygen. A chain reaction is thus started.

The radicals formed in the initiation and propagation steps may also react with each other. These types of reactions are called terminations. At a low dose rate, less R· and RO_2· will be formed in time and space, less terminations will occur and as a consequence more RO_2· will react with a neighbouring unsaturated fatty acid (*RH) when the same dose is applied. The extent of the chain reactions will then be enhanced and more lipids will be peroxidised at the lower dose rates.

4 Inverse dose rate effects as an indication that membrane damage is involved

The observation of an inverse dose rate effect in liposomes prompted us to examine Hb and K^+ leakage in erythrocytes after irradiation at different dose rates. Clear inverse dose rate effects were observed [7] for both end-points. An example of dose rate dependent Hb release is illustrated in figure 2.

To investigate dose rate effects on lymphocytes, these cells were isolated from bovine blood and irradiated at doses of 0.5, 1.0 and 1.5 Gy at dose rates of 0.361, 0.150, 0.062 and 0.006 Gy/min. The amount of trypan blue negative cells were counted directly after radiation as well as eight hours later. A considerable decrease of vital cells was observed [6] especially at the lower dose rates.

Encouraged by these results Ojeda and co-workers [13] performed experiments in which the presence of surface immunoglobin (S-Ig) of mouse lymphocytes was assayed after X-irradiation. They observed an inverse dose-rate effect with respect to radiation-induced loss of S-IgG expression. Chain reactions in the phospholipid bilayer of the membrane are probably the cause of damage to the receptors.

Membranes of living cells can be made more vulnerable for radiation induced damage by changing the saturated fatty acyl chains of phospholipids into poly-unsaturated fatty

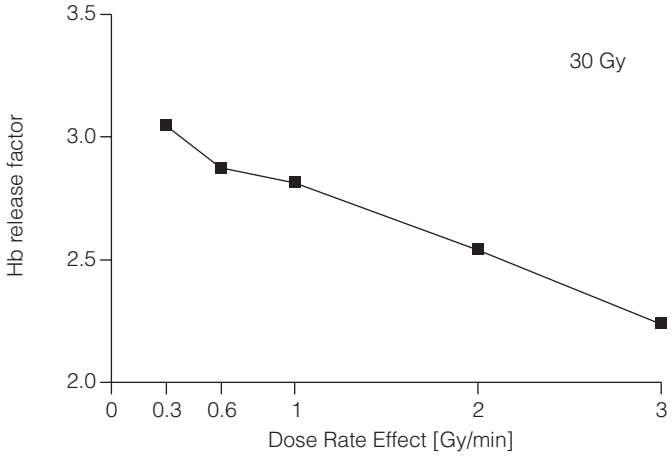

Fig. 2: X-irradiation of bovine erythrocytes. Hb leakage measured as published [7].

acyl chains. This has been performed by us [11, 22–25] with mouse fibroblasts grown in suspension in a serum free medium supplemented with arachidonic acid. This is a poly-unsaturated fatty acid (PUFA), very vulnerable to peroxidation [11, 12]. Although all membranes of these fibroblasts contained relatively high amounts of peroxidizable lipids, no effect on clonogenic ability after irradiation was observed when compared with the control cells. The radiation induced reproductive death of these cells showed a normal (sparing) dose rate effect. It is concluded that by using different dose rates for a certain radiation effect, indications for possible membrane involvement, as a cause for the damage, can be obtained.

5 Radiation damage to salivary glands; membrane involvement

In radiotherapy of head and neck cancers, salivary glands are often included in the field of irradiation, resulting in alterations of saliva excretion. Although salivary glands are composed of highly differentiated, almost noncycling, cells, these glands appear to be very vulnerable to radiation damage [19–21]. It was suggested that the high radiosensitivity of the salivary gland is related to the presence of secretory granules in the tissue at the time of irradiation. Damage to the membrane of the granula would then result in leakage of its proteolytic content into the cytoplasm, leading to the death of the cell. This possibility has been extensively studied by us during the last five years [4, 15–18].

When the membranes of the granules are the critical cellular targets then inverse dose rate effects have to be expected and degranulation of the cells prior to radiation should make the cells less radiosensitive. The latter situation is depicted in figure 3.

One of the parameters determining the extent of function loss is the duration of the lag phase. In our rat experiments this is the time period between subcutaneous injection of a secretion stimulus and the observation of the first response (collection) in terms of saliva excretion.

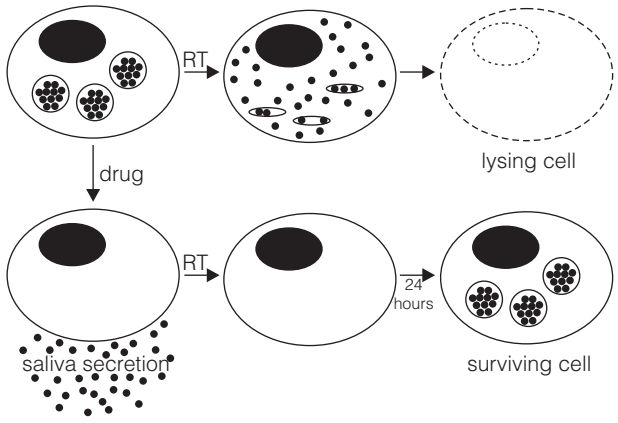

Fig. 3: The degranulation concept (for details see [4, 15, 16]).

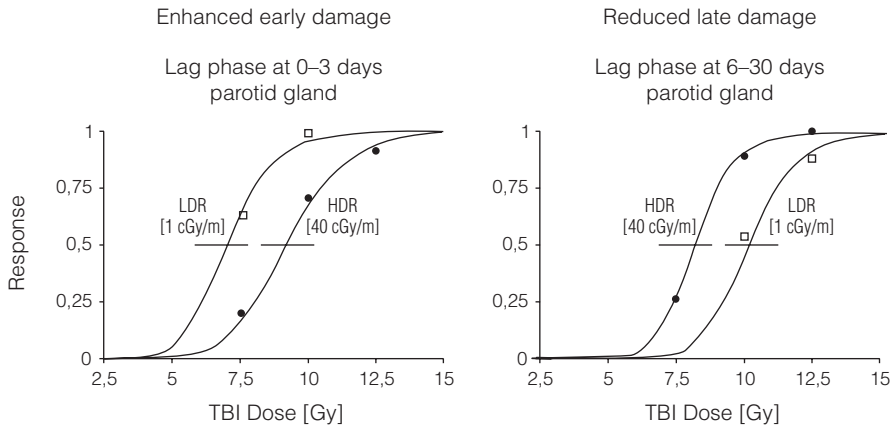

Fig. 4: γ-irradiation of rat salivary glands; dose rate effects (for details see [21]).

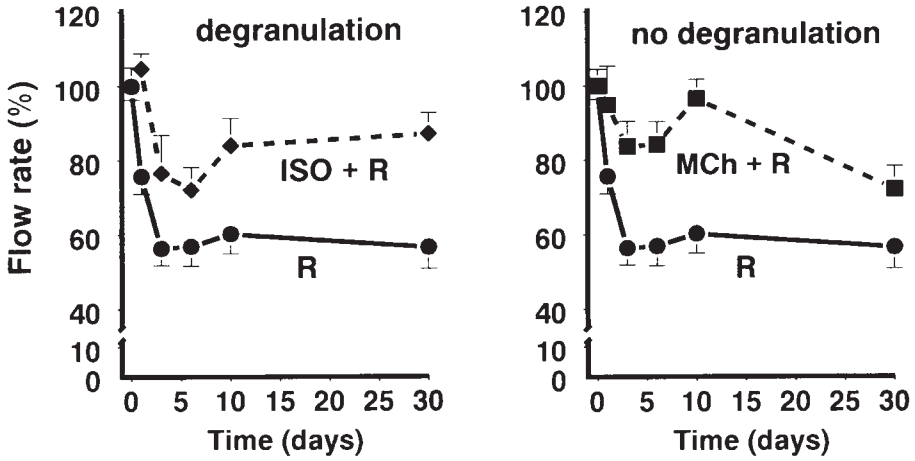

Fig. 5: X-irradiation of rat salivary glands. Importance of degrenulation for radioprotection [3, 4].

Rats were irradiated with a ^{60}Co-source. Total doses were 7.5, 10 and 12.5 Gy, delivered at dose-rates of 40 cGy/min (HDR) and 1 cGy/min (LDR). LDR irradiation significantly enhanced the increase of lag phase during days 0–3 [21]. At later times the reverse effect was seen with significant LDR sparing. This is illustrated in figure 4. So, although a membrane component is probably responsible for the early loss of function, the later effects seem to be caused by damage to other (non-membrane) structures. This means that leakage of granules cannot be the dominant cause of the observed function loss. To further investigate the "degranulation concept" it was decided to activate the parotid gland by different sialogogues. Ones that degranulate and ones that do not [3, 4, 18]. One result of such a comparison is given in figure 5. As can be seen both drugs protect

the cells against loss of function (flow rate) while only isoproterenol pretreatment had degranulated the cells. From these and other published [3, 4, 17] results it is concluded that the granules are not the main targets for radiation induced function loss of the parotid gland.

6 Receptor coupled signal transduction pathways in the salivary gland

The principal regulatory mechanism for salivary secretion is via neurotransmitter release by innervated sympathetic (adrenergic) and parasympathetic (cholinergic) nerves of the automatic system. Neurotransmitters can activate appropriate cell surface receptors on the basolateral membrane. The main receptors of the acinar cells in the parotid gland are α- and β-adrenergic and muscarinic-cholinergic. Translation into a physiological response of the signal initiated at the receptor, involves a signal transduction mechanism via GTP-binding proteins. These G proteins, situated on the intracellular side of the plasmamembrane, are connected to the integral membrane proteins (seven membrane-spanning regions) of the receptor. Intracellular signaling is conducted via the G proteins to effector molecules such as adenylate cyclases and phospholipases or directly to ion channels or kinase functions. Elucidation of the the signalling pathways connecting the surface receptors to nuclear events, have only just begun. These pathways involve a number of biochemical routes including molecules regulating kinase cascades. The salivary gland β-adrenoceptor exerts its effect through activation of adenyl cyclase, formation of cAMP, and activation of cAMP-dependent protein kinase A (PKA). The α-adrenoceptor and the muscarinic (cholinergic) receptor can activate phospholipase C (PLC), which hydrolyses phosphatidyl-inositol-4,5-bisphosphate (PIP2), subsequently leading to the formation of two second messengers,

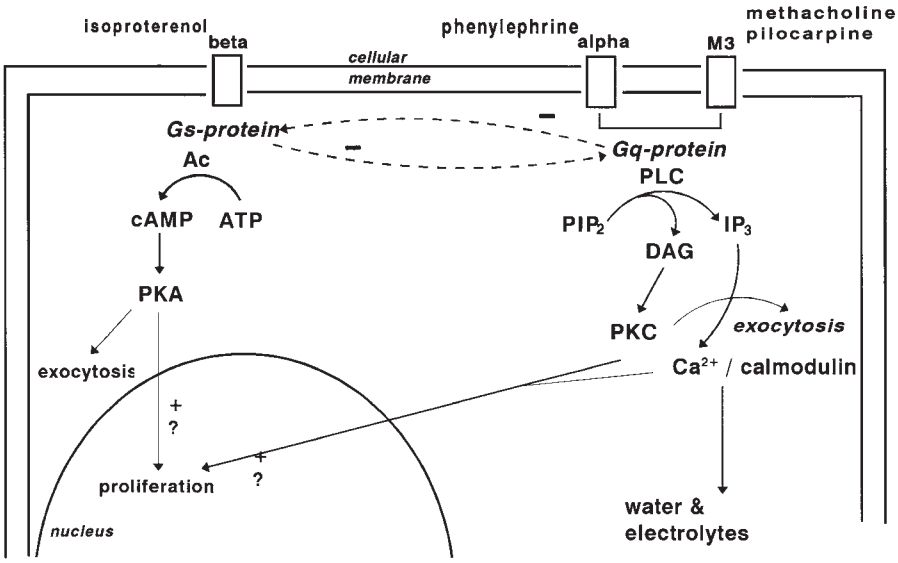

Fig. 6: Schematic representation of receptor activities in the acinus of rat parotid gland.

namely inositol-4,5-triphosphate (IP3), which mobilizes Ca^{2+} from intracellular stores and diacylglycerol (DAG), which activates protein kinase C (PKC). Figure 6 is a schematical illustration of these systems in the parotid acinar cell. Secretion of water and electrolytes is induced via the α-adrenoceptor and the cholinergic muscarinic (M) receptor. Protein secretion is mainly via the β-adrenoceptor.

7 Cellular radioprotection by receptor activation

Recent studies in our laboratory have indicated [14] that the high radiosensitivity of salivary glands is not accompanied by substantial cell killing. In fact, only very low radiation induced apoptosis could be detected and no significant cell loss was seen up to 10 days after a dose as high as 15 Gy, while gland function had decreased considerably. From these experiments it was concluded that radiation had disabled the cells to function properly in terms of saliva secretion, but that they were not killed to an extended degree.

Several agonists have been tested for their potential to protect the parotid and the submandibular gland of the rat against radiation damage [3, 4, 18]. The table shows the results after pretreatment with single sialogogues as well as with some combinations on the function of the parotid gland. As can be seen (+ and ++), activation of either receptor can lead to a protected status of the cells. Stimulation of both the α-and the M-receptor together gives the best protection on the long run (up to 240 days). They both stimulate secretion via the PIP2/PLC pathways. The opposite is true for a combination that stimulates the PIP2/PLC pathway together with the AC/cAMP pathway. The latter phenomenon can be explained [3] by inhibitory cross talk between those two second messenger systems. For future clinical application a combination of non-toxic drugs that activate the α- and the M-receptor should be tested.

Table 1: Radiation protection by sialogogues pretreatment.

			Parotid gland function			
Drug	Receptor	Sec. Mess.	0–6 days	6–30 days	30–120 days	120–240 days
Phenylephrine	α_1-AR	PIP_2/PLC	0	++	+	0
Isoproterenol	β-AR	AC/cAMP	+	++	+	0
Methacholine	MAchR (M3)	PIP_2/PLC	++	++	0	0
Pilocarpine	MAchR (M3)	PIP_2/PLC	++	++	0	0
Methacholine Phenylephrine	MAchR α_1-AR	PIP_2/PLC PIP_2/PLC	+	++	++	++
Methacholine Isoproterenol	MAchR β-AR	PIP_2/PLC AC/cAMP	0	0		
Isoproterenol Phenylephrine	β-AR α_1-AR	AC/cAMP PIP_2/PLC	0	0		

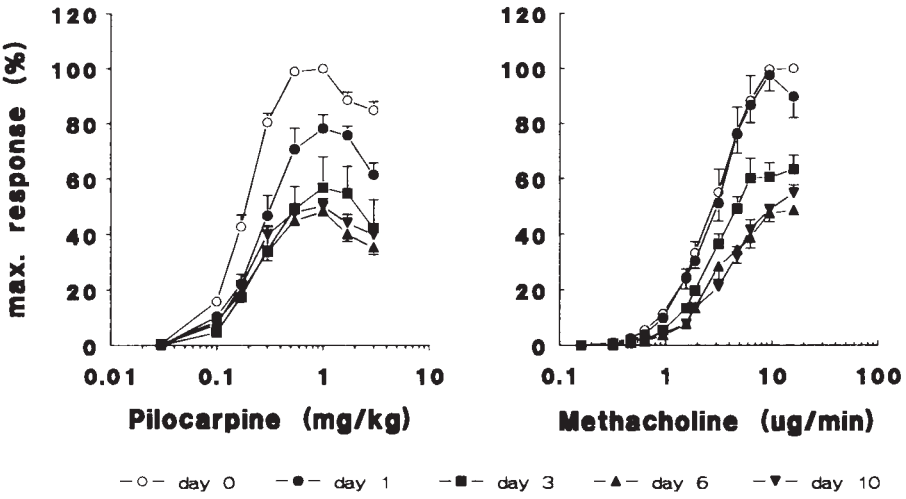

Fig. 7: X-irradiation of rat parotid gland. Effect of radiation damage on saliva stimulation by a partial (Pilo) and full (MC) agonist of the M3-receptor.

8 Mode of action

Activation of α-, or β-adrenergic and M-cholinergic receptors can protect the acinar cells of the parotid gland against radiation damage. The type of damage is not known at the moment. For the very early effects (0–3 days) a membrane component seems to be involved [21], as has been discussed above (inverse dose rate effect). Therefore ligand binding and the efficiency of coupling between the receptor and the G protein was investigated for the M-cholenergic receptor. Binding of agonists to the receptor as well as coupling efficiency of the receptor to the G protein, prior to and days after irradiation, were assayed for the partial agonist pilocarpine (Pilo) in comparison to the full agonist methacholine (MC). Pilo uses the full capacity of the receptor-G protein coupling, while MC does not. So for MC there exists a so called "G-protein reserve" capacity. This agonist dependent difference in activation of the starting point of the same second messenger system, can be used to obtain information on possible radiation damage to the receptor G protein coupling. Moderate radiation damage to the coupling system may lead to less stimulation of the fluid phase of saliva by Pilo, while stimulation by MC may not be altered because of the use of the G protein reserve capacity. This type of experiments, supplemented with the necessary controls, have been performed recently [2]. Figure 7 illustrates the percentage of the maximal response on saliva flow rate in the parotid gland of the rat by different concentrations of Pilo or MC at day 0, 1, 3, 6, and 10 after a radiation dose of 15 Gy of X-rays. As can be seen in this figure saliva stimulation by Pilo is clearly diminished already one day after the radiation. This however is not the case when the saliva is stimulated by MC. From this result we conclude that radiation has probably damaged the coupling between the M-receptor and the G protein. The data cannot be explained by compromised ligand binding of Pilo to the receptor, because the binding efficiency is not altered (constant ED50). At day three after the radiation also the MC stimulated flow rate is inhibited. For both drugs the effect is maximal at day 6.

This further decline indicates that the cell's signal transduction system deteriorates when time proceeds. At the moment we hypothesize that the early radiation effect, within 3 days, is membrane damage affecting the receptor G protein coupling, explaining the earlier observed [21] inverse dose rate effect. Later damage then is predominantly of non membrane nature (sparing by low dose rates) and located in the cytoplasm downstream the G protein in the second messenger signal transduction system.

9 Concluding remarks

In this paper a short overview has been given on a certain aspect of the research program performed at the Department of Radiobiology of the University of Groningen during the last 25 years. The topic was restricted to possible membrane involvement in cellular dysfunction and cell death after ionizing radiation. It shows how idea's concerning mechanisms of action, developed ten or more years ago, can be incorporated into modern approaches of research. It is a challenge for the near future to find out if the observed transient cellular protection afforded by agonist binding to G protein coupled receptors in salivary glands is of a more general nature and how the protection is realised at the molecular level.

10 Literature

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